Decarboxylation of Bovine Prothrombin Fragment 1 and Prothrombin†

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ABSTRACT: Bovine prothrombin fragment 1 and prothrombin undergo decarboxylation of their γ-carboxyglutamic acid residues when the lyophilized proteins are heated in vacuo at 110 °C for several hours. The fully decarboxylated fragment 1 product has lost its barium-binding ability as well as the calcium-binding function which causes fluorescence quenching in the presence of 2 mM Ca^{2+}. There is no sign of secondary structure alteration in solution upon analysis by fluorescence emission and circular dichroic spectroscopy. A family of partially decarboxylated fragment 1 species generated by heating for shorter time periods shows that the initial decrease in calcium-binding ability occurs almost twice as rapidly as the loss of γ-carboxyglutamic acid. This is consistent with the idea that differential functions can be ascribed to the 10 γ-carboxyglutamic acid residues in fragment 1, including both high- and low-affinity metal ion binding sites. Prothrombin itself also undergoes total decarboxylation without any apparent alteration in secondary structure. However, in this case the latent thrombin activity is progressively diminished during the heating process in terms of both clotting activity and hydrolysis of the amide substrate H-D-Phe-Pip-Arg-pNA. The present results indicate that in vitro decarboxylation of γ-carboxyglutamic acid in dried proteins is useful for analyzing the detailed calcium-binding properties of vitamin K dependent coagulation factors.

In the final stage of blood coagulation, prothrombin is activated to thrombin, and the formation of the activation complex requires the presence in the zymogen of an unusual amino acid, γ-carboxyglutamic acid. Prothrombin contains 10 Gla[1] residues, all of which are situated in fragment 1, the NH_2-terminal activation peptide (Fernlund et al., 1975; Magnusson et al., 1975; Howard et al., 1975). A vitamin K dependent reaction is responsible for the carboxylation of Glu to Gla following prothrombin polypeptide chain biosynthesis (Suttie & Jackson, 1977). The study of abnormal prothrombins isolated from animals treated with vitamin K antagonists such as dicoumarol (Stenflo & Ganrot, 1972; Nelsestuen & Suttie, 1972) led to clarification of the nature and functional significance of γ-carboxyglutamic acid in normal prothrombin. Chiefly, Gla is essential for the interaction of prothrombin with phospholipid membranes which is mediated by Ca^{2+} binding (Esmon et al., 1975; Stenflo, 1973; Nelsestuen & Suttie, 1973). Related coagulation proteins, including factors X and IX and protein C, were shown to contain Gla in homologous sequence positions (Stenflo & Suttie, 1977), and another protein, the Gla protein from calf bone, was also discovered to contain Gla (Hauschka et al., 1975; Price et al., 1976). NMR analysis has uncovered some specific metal ion binding properties of Gla and its derivatives (Sperling et al., 1978; Robertson et al., 1978).

Learning that γ-carboxyglutamic acid plays a role in Ca^{2+} binding to prothrombin has been accompanied by studies of the effect this binding produces on the molecule in solution. Our laboratory has proposed a model for prothrombin fragment 1–metal ion interactions in which two classes of metal ion binding sites can be distinguished in terms of their function (Bloom & Mann, 1978). The binding of Ca^{2+} in the first class of sites is relatively nonselective and causes a conformational change in the molecule which can be observed on fluorescence and circular dichroic analyses. The filling of a second class of sites by Ca^{2+} is related to dimerization of fragment 1 molecules and to the lipid-binding process associated with the transformation of prothrombin to thrombin. A necessary inference of this model is that differential functions can be assigned among the 10 Gla residues in fragment 1.

Further study of dicoumarol-induced abnormal prothrombins has been leading to a more detailed picture of how γ-carboxyglutamic acid is involved in Ca^{2+} binding and the activation pathway. Early experiments suggested the existence of a range of defective prothrombins which bound to barium salts with varying affinity (Malhotra, 1972; Morrison & Esnouf, 1973). After Gla became recognized, it was apparent that dicoumarol-treated bovine and human plasma contained not just one completely noncarboxylated prothrombin but rather a whole series of partially carboxylated products (Esnouf & Prowse, 1977; Friedman et al., 1977). For example, one warfarin-affected prothrombin with 66% Gla exhibited reduced biological activity, while another with 40% Gla had practically no activity (Esnouf & Prowse, 1977). We are interested in pursuing the problem from an additional clinical standpoint. For patients undergoing vitamin K antagonist therapy, the question of the carboxylation state of prothrombin is important in assessing the risk of concurrent hemostatic failure.

The impaired binding properties of abnormal prothrombins make it difficult to isolate purified materials for analysis from plasma (Prowse et al., 1976). This paper reports the use of a synthetic decarboxylation procedure to create a series of partially decarboxylated bovine prothrombin and fragment 1 species which presumably resemble the partially carboxylated proteins from animal sources. We have correlated the carboxylation state of the Gla residues with some physical, spectroscopic, and biological properties of the molecules. Fully decarboxylated materials exhibit no additional structural alterations but suffer the loss of functional aspects related to binding and catalysis. While this work was in progress, Poser & Price (1979) reported on the decarboxylation of calf bone Gla protein using a procedure based upon identical principles.

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[1] Abbreviation used: Gla, γ-carboxyglutamic acid.

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Experimental Section

Proteins. Prothrombin was prepared from citrated bovine plasma as previously described (Bajaj & Mann, 1973). Prothrombin fragment 1 was prepared by proteolytic cleavage of prothrombin by thrombin as described elsewhere (Heldebrant et al., 1973). Both protein preparations were greater than 95% homogeneous upon examination by gel electrophoresis. Bovine factor Xa and factor Va were prepared as previously described (Downing et al., 1975; Nesheim et al., 1979). An abnormal prothrombin from warfarin-treated cows was kindly provided by Dr. Bruce Furie, Tufts University; it did not contain detectable Gla (Furie, B., et al., 1978). Protein concentrations were determined spectrophotometrically at 280 nm and corrected for Rayleigh scattering. Extinction coefficients of 14.4 and 10.5 were used for prothrombin and fragment 1, respectively (Mann & Elion, 1978). Proteins were examined by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis according to the method of Weber & Osborn (1969) as modified by Mann et al. (1971).

Materials. Synthetic γ-carboxyglutamic acid was a gift from Dr. Barbara Furie, Tufts University. Aluminum hydroxide was prepared according to Quick (1951). Phospholipid vesicles were prepared as described elsewhere (Bloom et al., 1979). Venom from *Echis carinatus* was obtained from Sigma. The thrombin substrate H-D-Phe-Pip-Arg-pNA·2HCl (S-2238) was obtained from Ortho Diagnostics. Milli-Q water (Millipore Corp.) used throughout was decalcified to the extent of $[Ca^{2+}] = 6.4 \times 10^{-9}$ M.

Decarboxylation. In preparation for reaction, the proteins were dialyzed in plastic containers first against 10 mM EDTA–0.02 M Tris-HCl–0.15 M NaCl, pH 8.4, to ensure minimal Ca^{2+} contamination and then exhaustively against water at 5 °C. After lyophilization, 5–10 mg of dry fragment 1 or prothrombin was placed in a hydrolysis tube. The protein was alternately evacuated and flushed via a three-way stopcock connected to a flask containing nitrogen saturated with acetic acid vapor and then thoroughly evacuated. Decarboxylation was effected by heating the protein in vacuo at 110 °C in a block heater for various periods of time. After being heated, the protein was suspended in 1 mL of water and apportioned into aliquots for lyophilization and analysis.

Amino Acid Analysis. Alkaline hydrolysis of analysis samples was carried out in 2 N KOH in evacuated glass tubes for 24 h at 110 °C (Hauschka, 1977). Amino acid analyses were performed on a Beckman 119 amino acid analyzer utilizing Beckman AA15 resin. For the analysis of Gla, samples were applied in sodium citrate buffer adjusted to pH 1.0 with HCl (Fernlund et al., 1975) and the column was developed with 0.2 M sodium citrate, pH 3.26. A standard γ-carboxyglutamic acid sample eluted at 38 min between cysteic acid (20 min) and aspartic acid (51 min). The extent of decarboxylation among the heated proteins was measured by a combination of the decrease in the Gla peak and a corresponding increase in the Glu peak.

Binding Studies. Fragment 1 was tested for selective adsorption to barium citrate and aluminum hydroxide (Malhotra, 1972). A solution of decarboxylated fragment 1, ~0.2 mg/mL in 0.04 M sodium citrate (pH 8.8), was made 0.06 M in $BaCl_2$, and after the solution was stirred for 1 h the barium citrate precipitate was removed by centrifugation. To the supernate was added aluminum hydroxide slurry (1/15 volume), and after the solution was stirred for 1 h more the aluminum hydroxide was removed by centrifugation. The extent of binding among the heated proteins was measured by the decrease in solution protein concentration. Fragment 1 was almost completely eluted from both barium citrate and aluminum hydroxide by stirring in 0.2 M sodium citrate–0.15 M NaCl, pH 9.0.

Fluorescence Measurements. The fluorescence intensities of prothrombin and fragment 1 were recorded on a Perkin-Elmer MPF-44A spectrophotometer equipped with a DCSU-2 differential corrected spectra unit. In preparation for analysis, decarboxylated protein samples were first dialyzed against 10 mM EDTA as above and then into 0.01 M Tris-HCl–0.10 M NaCl, pH 7.5, and solutions were filtered through a 0.45-μm Millipore filter. All measurements were made at 25 °C with excitation at 295 nm and emission scanning from 270 to 380 nm encompassing the peak of interest with λ_{max} 332 nm. Titrations of fluorescence intensity were performed in 2.5-mL volumes containing ~0.1 mg/mL protein by adding Ca^{2+} to a final concentration of 2 mM and allowing the solution to equilibrate for 5 min after mixing (Prendergast & Mann, 1977). The difference in the relative fluorescence intensities of the two solutions, untitrated (F_0) and titrated (F_∞), was determined by comparing values at λ_{max} despite a blue shift of 2 nm for F_∞. Average readings of duplicate spectra are reported.

Circular Dichroism Measurements. Circular dichroism (CD) spectra of fragment 1 were recorded on a Jasco Model J-20A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The far-UV spectra were measured in 3.0-mL volumes containing 0.2–0.3 mg/mL protein at 25 °C. Base line corrected CD spectra were derived for native and decarboxylated fragment 1 in the absence and presence of 5 mM Ca^{2+}. Average readings of duplicate spectra are reported in terms of mean residue ellipticity, $[\theta']$.

Prothrombin Activation. Prothrombin was activated by the action of either a factor Xa system or a proteolytic snake venom. In the former case, normal or decarboxylated prothrombin at 0.1 mg/mL in 0.01 M Tris-HCl–0.10 M NaCl, pH 7.5, was incubated with the following activators: 1 μg/mL factor Xa, 5 μg/mL factor Va, 2 mM Ca^{2+}, 0.25% phospholipid (final concentrations). Reaction was continued at 37 °C until maximal thrombin activity was generated, never longer than 1 h. Alternatively, prothrombin was activated by treatment with *E. carinatus* venom (0.1 mg/mL) at pH 7.5 and 37 °C until constant thrombin activity was attained, typically 3 to 4 h (Nelsestuen & Suttie, 1972). A clotting assay was used to monitor thrombin production (Mann, 1976). The amidolytic activity of thrombin was tested by following the hydrolysis of the substrate H-D-Phe-Pip-Arg-pNA (S-2238) spectrophotometrically at 405 nm (Bang & Mattler, 1977).

Results

The first indication that a synthetic method for decarboxylation of γ-carboxyglutamic acid could successfully be adapted to Gla residues in lyophilized proteins came to us with the observation that prothrombin fragment 1 was unchanged in physical appearance and completely soluble in common buffers even after heating in vacuo for 96 h. Prothrombin itself also survived reaction without change in physical appearance except for a slight amount of insoluble material left upon dissolution in buffers.

The techniques described here for pretreatment and decarboxylation were selected with the aim of keeping the proteins as close as possible to their "native" condition. Fragment 1 and prothrombin were dialyzed against water to reduce the likelihood of conformational changes induced by pH extremes and to ensure a minimum of buffer salts in lyophilized material. In early experiments fragment 1 was fully decarbox-

ylated by heating for 6 h after lyophilization from 0.25 M acetic acid, pH 3.0, or 0.25 M NH_4OH, pH 10.6, as well as from water. However, traces of HCl are damaging, as reaction of fragment 1 after lyophilization from 0.05 M HCl, pH 1.6, led to the production of a charred insoluble material. Reaction of fragment 1 after lyophilization from water and subsequent exposure to HCl vapor led to unpredictable amounts of fragmentation of the polypeptide chain as viewed on Na-$DodSO_4$ gels. Although fragment 1 was routinely decarboxylated after preexposure to acetic acid vapor, this step was found to be unnecessary except to increase the reaction rate by ~20%.

Amino acid analysis for direct quantitation of Gla and Glu provided a reliable measure of the extent of decarboxylation among the heated proteins, against which to compare their Gla-dependent functions. Application of hydrolysates to the column in sodium citrate buffer, pH 1.0 (Fernlund et al., 1975), yielded an isolated symmetrical Gla peak amenable to quantitation and containing more than 90% of the Gla present. Fragment 1, which has been heated for 6 h at 110 °C, is arbitrarily referred to as "fully decarboxylated" even though 9% Gla does remain (Figure 3). One sample heated for 24 h did not yield 0% Gla remaining, and the reason for this is unclear but may be related to protein dimerization.

Electrophoretic analysis of decarboxylated fragment 1 and prothrombin in 0.1% $NaDodSO_4$ on 10% polyacrylamide gels, pH 7.2, was undertaken to test for higher or lower molecular weight materials, pointing to polymerization or degradation. A gel of a mixed sample consisting of normal and fully decarboxylated fragment 1 showed a closely spaced doublet with the latter migrating farther toward the anode. Gels of decarboxylated protein exhibited no bands migrating farther than the single major band, indicating no peptide bond cleavage during the heating process. However, in gels of decarboxylated fragment 1 a faint band appeared at a position corresponding to a dimer. The barium-binding ability of fragment 1 was also eliminated by decarboxylation as expected. Fully decarboxylated fragment 1 did not bind appreciably (8%) to barium citrate but did bind strongly (94%) to aluminum hydroxide (Figure 3).

Comparison of Normal and Fully Decarboxylated Fragment 1. Two different spectroscopic techniques, fluorescence and circular dichroism (CD), were employed to compare the structural properties of normal and fully decarboxylated fragment 1. In these experiments three kinds of bovine fragment 1 were used, designated as follows: "native", dialyzed only; "normal", dialyzed and lyophilized; "decarboxylated", normal protein subjected to fully decarboxylating conditions (heating for 6 h at 110 °C).

Figure 1 depicts the fluorescence spectra of normal fragment 1 (solid line) and fully decarboxylated fragment 1 (dashed line) in the absence of Ca^{2+} (left half). The peaks are identical in appearance and shape, supporting our contention that the decarboxylation process does nothing else to alter the solution conformation of the molecule, insofar as is detectable by the tryptophan fluorophores. In a separate experiment, a solution of decarboxylated fragment 1 was made 6 M in guanidine hydrochloride. Under this denaturing condition the fluorescence peak was substantially altered both in broadened shape and shifted λ_{max} to 345 nm. This implies that decarboxylated fragment 1 has not suffered any thermal denaturation. The only noticeable difference in the fluorescence peaks is a small displacement in λ_{max} from 332 nm for normal to 334 nm for decarboxylated. A satisfactory correlation existed between fluorescence intensity at 332–334 nm and UV absorbance at

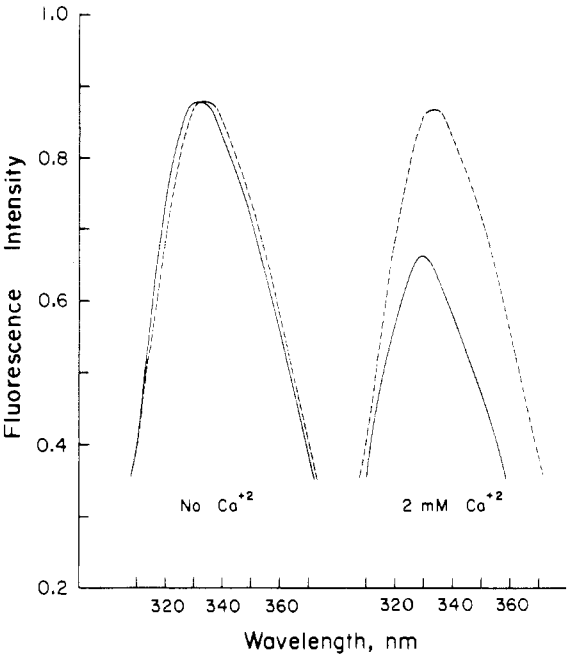

FIGURE 1: Fluorescence intensity spectra of bovine prothrombin fragment 1 (—) and fully decarboxylated fragment 1 (- - -) in the absence (left) and presence (right) of 2 mM Ca^{2+}. Protein concentrations were 0.1 mg/mL. Excitation was at 295 nm, and emission was recorded as relative fluorescence intensity. No further quenching occurred upon Ca^{2+} addition beyond 2 mM.

280 nm, thus promoting confidence in the use of absorbance at 280 nm as a device for measuring decarboxylated fragment 1 concentration in solution.

Bovine fragment 1 is known to undergo fluorescence quenching in the presence of Ca^{2+}; the quenching is complete at 1 mM Ca^{2+} and occurs to a maximum extent of −46% at 340 nm (Bloom & Mann, 1978; Prendergast & Mann, 1977; Nelsestuen, 1976). This phenomenon is associated with the binding of Ca^{2+} to fragment 1 which induces a conformational change that is expressed as a decrease in fluorescence intensity at λ_{max}. Figure 1 illustrates the fluorescence quenching of normal fragment 1 (solid line) and decarboxylated fragment 1 (dashed line) in the presence of 2 mM Ca^{2+} (right half). For normal fragment 1 as well as native (not shown) the maximum extent of quenching was −25%, which is explained by the finding that fragment 1 exhibits reduced quenching after periods of storage (Nelsestuen, 1976). For fully decarboxylated fragment 1 we find that Ca^{2+}-dependent fluorescence quenching is essentially abolished, occurring to a maximum extent of only −1%. This is to be expected if the presence of Gla is responsible for fragment 1 calcium binding. Again, one noticeable difference upon titration of the fluorescence peaks is a slight change in the properties of λ_{max}; for normal it shifts from 332 to 330 nm, while for decarboxylated it remains at 334 nm.

When fragment 1 binds Ca^{2+}, the same conformational change which leads to fluorescence quenching at λ_{max} also leads to a significant change in the appearance of the circular dichroic spectrum (Bloom & Mann, 1978). Figure 2 depicts the CD spectra of normal fragment 1 (solid line) and 24-h decarboxylated fragment 1 (dashed line) both in the absence and presence of 5 mM Ca^{2+}. The spectra are identical within experimental error in the absence of Ca^{2+}, providing the strongest evidence that the decarboxylation process does nothing else to alter the solution conformation of the molecule in terms of gross secondary structure. Upon addition of Ca^{2+} to 5 mM, the CD spectrum of normal fragment 1 shifts to

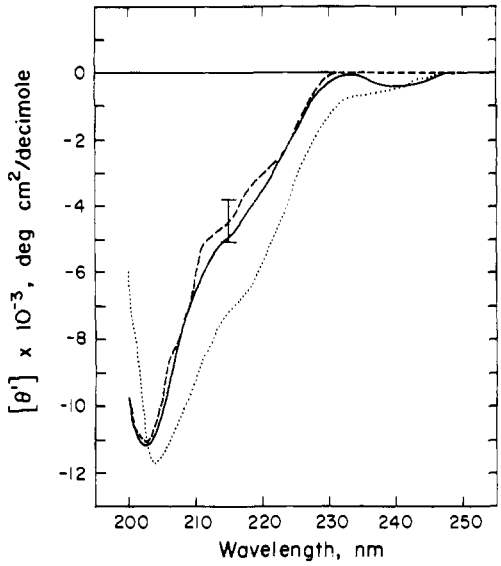

FIGURE 2: Far-ultraviolet CD spectra of bovine prothrombin fragment 1 in the absence (—) and presence (···) of 5 mM Ca^{2+} and of fully decarboxylated fragment 1 in the absence (- - -) and presence (not shown) of 5 mM Ca^{2+}. The latter is not shown for clarity because it is virtually superimposable on the spectrum of fully decarboxylated fragment 1 in the absence of Ca^{2+}. Protein concentrations were 0.2–0.3 mg/mL. [θ'] is the mean residue ellipticity.

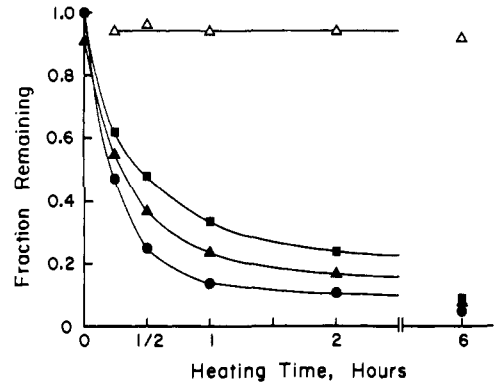

FIGURE 3: Decarboxylation of bovine prothrombin fragment 1 by heating at 110 °C and the effect on various Gla-dependent characteristics. The fraction of γ-carboxyglutamic acid remaining in fragment 1 (closed squares), the fraction of fragment 1 binding to barium citrate (closed triangles) or aluminum hydroxide (open triangles), and the fraction of fragment 1 capable of undergoing fluorescence quenching by 2 mM Ca^{2+} (closed circles) are plotted vs. heating time.

longer wavelength (dotted line) while that of decarboxylated fragment 1 remains unchanged (superimposable on dashed line). Thus, the loss of Gla from fragment 1 prevents calcium binding, in agreement with the fluorescence results.

Analysis of Partially Decarboxylated Fragment 1. The influence of incomplete decarboxylation on two Gla-dependent characteristics of fragment 1, barium binding and calcium binding, was examined in order to correlate the partial carboxylation state with functional properties of the protein. Figure 3 depicts the time-dependent decarboxylation of Gla residues (closed squares) in comparison to the time-dependent losses of barium-binding ability (closed triangles) and calcium-binding ability (closed circles) of the protein.

The results for actual decarboxylation of fragment 1 are displayed in Figure 3 (closed squares), where "fraction remaining" represents γ-carboxyglutamic acid left after reaction. A family of partially decarboxylated fragment 1 species was generated by heating in vacuo at 110 °C over time

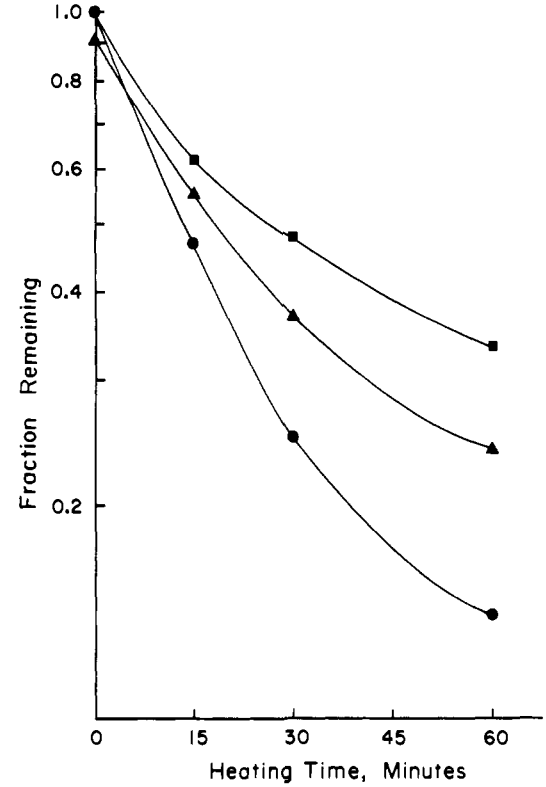

FIGURE 4: Semilogarithmic plot of the fractional changes in γ-carboxyglutamic acid content (closed squares), barium citrate binding (closed triangles), and fluorescence quenching (closed circles) vs. heating time.

periods ranging from 0 to 6 h. The approximate half-time of this reaction was 27 min. The results for barium binding (closed triangles) refer to binding of fragment 1 to barium citrate; here $1 - (A/A_0)$ is plotted vs. time, where A is the unbound protein concentration measured by absorbance at 280 nm. A progressive lack of ability to bind to barium citrate accompanied the slower progressive loss of Gla from fragment 1. By contrast, binding to aluminum hydroxide (open triangles) was not affected by any level of decarboxylation. The results for calcium binding (closed circles) refer to fluorescence quenching of fragment 1 in the presence of 2 mM Ca^{2+}; here $1 - (F_0/F_\infty)$ is plotted vs. time, where F_0 and F_∞ are the fluorescence intensities of the peak at 332 nm in the absence and presence of 2 mM Ca^{2+}, respectively; fractions from 0 to 1 correspond to quenching from 0% to 25% (observed maximal). A progressive lack of fluorescence quenching upon addition of Ca^{2+} accompanied the slower progressive loss of Gla from fragment 1. The approximate half-time of this process was 14 min.

Figure 4 depicts the portion of the data in Figure 1 during the first hour of reaction replotted on a semilogarithmic basis. A marked deviation from linearity in the loss-of-Gla curve (closed squares) suggests that the decarboxylation of fragment 1 is not a simple first-order process, even during the initial phase. Likewise, the curves for barium citrate binding (closed triangles) and fluorescence quenching by Ca^{2+} (closed circles) reveal that neither decrease is strictly a first-order process. For a comparison of the three Gla-dependent characteristics of fragment 1 shown in Figure 4 on a semiquantitative basis, estimated rate constants were derived for the initial loss of these Gla-dependent functions. This was accomplished by taking the first three points of each curve in Figure 4, calculating the slope of the best-fitting straight line, and assuming the relative slope to be an approximate first-order rate constant

Table I: Decarboxylation of Bovine Prothrombin and Activation to Thrombin

prothrombin[a]	decarboxylation[b] Gla remaining	rel	sp act. (NIH units/mg)[c] factor Xa[e]	rel	venom[e]	rel	S-2238 hydrolysis[d] [$\Delta A(405)$/(min mg)] venom[e]	rel
native			1213		1288		175	
normal	10.0	1	810	1	824	1	177	1
110 °C, 1 h	3.1	0.31	593	0.73	699	0.85	116	0.65
110 °C, 6 h	0.8	0.08	271	0.33	254	0.31	45	0.26
110 °C, 22 h			39	0.05	62	0.08	44	0.25
abnormal	<0.5	<0.05	177	0.22	791	0.96	168	0.95

[a] Prothrombin types: native, dialyzed only; normal, lyophilized; 110 °C, decarboxylated; abnormal, from warfarinized cows. [b] Number of Gla determined by amino acid analysis. [c] Prothrombin activatibility determined by clotting assay of thrombin produced. [d] Thrombin amidolytic activity against the substrate H-D-Phe-Pip-Arg-pNA. [e] Activation systems: factor Xa, factor Va, Ca^{2+}, and phospholipid system and *E. carinatus* venom system; described under Experimental Section.

for the initial decay process. The values of k for fragment 1 are as follows: loss of Gla (decarboxylation), 1.5 h^{-1}; loss of barium citrate binding, 1.8 h^{-1}; loss of fluorescence quenching, 2.8 h^{-1}.

Prothrombin Decarboxylation and Activation. Experiments analogous to those using fragment 1 were performed on the whole prothrombin molecule. In addition to the three kinds of bovine prothrombin used, designated as before native, normal, and decarboxylated, another kind was also used, "abnormal", isolated from warfarin-treated animals and known to possess neither detectable γ-carboxyglutamic acid nor biological activity (Furie, B., et al., 1978).

Bovine prothrombin which has been heated for 6 h at 110 °C is again referred to as "fully decarboxylated" even though 8% Gla does remain (Table I), and another sample heated for 22 h can also be designated as such. A typical fluorescence experiment demonstrated that the peaks representing normal and fully decarboxylated prothrombin were identical in appearance. The λ_{max} for both peaks was 330 nm. For the decarboxylated prothrombins we observed a qualitative absence of fluorescence quenching in the presence of Ca^{2+}. One partially decarboxylated prothrombin species produced by heating for 1 h at 110 °C showed that the half-time of the reaction was similar to that with fragment 1. A fluorescence experiment was technically unable to correlate partial decarboxylation with reduced fluorescence quenching upon addition of Ca^{2+}. This is not surprising since normal prothrombin undergoes quenching to a maximum extent of only −6% (Nelsestuen, 1976) and we observed a value of −4%.

Prothrombin activation experiments were carried out to test the hypothesis that the COOH-terminal thrombin region of the molecule remains intact upon decarboxylation. The results indicate that it does not. It was necessary to study the activatibility of decarboxylated prothrombin by using two separate methods, one which requires Gla and one which does not. The first was a factor Xa system, the physiological "prothrombinase" complex, consisting of factor Xa, factor Va, Ca^{2+}, and phospholipid; it requires Ca^{2+} and the presence of Gla on the prothrombin substrate for activation. The second was a snake venom system, *E. carinatus* venom, a nonphysiological, prothrombin-specific activator which requires neither Ca^{2+} nor Gla for activation (Franza et al., 1975). All prothrombin preparations were activated to a quantifiable degree as listed in Table I. A fibrinogen clotting assay was used to measure specific activity in NIH units per milligram. Factor Xa activations proceeded fairly rapidly; normal prothrombin was complete within 5 min and fully decarboxylated prothrombin reached a constant level within 1 h, and the values reported are plateau levels. By contrast, venom activations proceeded much more slowly; all prothrombins took at least 3 h to reach a constant level, and the values reported were taken at 4 h. Electrophoretic analysis of venom-activated prothrombin samples on $NaDodSO_4$ gels confirmed that starting material was completely converted into the usual set of activation fragments, even in the case of fully decarboxylated material.

The first noteworthy observation is that normal prothrombin was only ~66% as activatible as native prothrombin in either system; hence, the effect of lyophilization alone reduced the potential thrombin by about one-third. Normal prothrombin was selected as the control for the rest of the samples. Abnormal prothrombin was almost totally activatible by venom (96%) while quite poorly so by factor Xa (22%), in agreement with the original results of Nelsestuen & Suttie (1972). Among the decarboxylated prothrombins, both venom and factor Xa activations yielded only partial activatibility, ranging down to as low as 5%. There is only an incidental correlation between the decrease in carboxylation state of prothrombin and the decrease in incipient thrombin as determined by clotting ability. In a separate experiment also listed in Table I, venom-activated prothrombin was analyzed in terms of the rate of thrombin hydrolysis of the nitroanilide substrate H-D-Phe-Pip-Arg-pNA. Abnormal prothrombin again acted almost as well as normal (95%) whereas decarboxylated material yielded thrombin showing a comparatively low hydrolysis rate (25%). The most significant observation is that fully decarboxylated prothrombin, unlike abnormal, cannot be activated by the venom system to any great degree (less than 10%). Thus, it appears that the decarboxylation process not only destroys the Gla residues in the fragment 1 region but also exerts enough impact on the thrombin region to eliminate latent enzymatic activity in the zymogen.

Discussion

Thermally induced decarboxylation of γ-carboxyglutamic acid residues may be anticipated in view of the well-known tendency of malonic acid derivatives to lose 1 equiv of CO_2 under conditions of acidic heating (March, 1977). The synthetic decarboxylation of γ-carboxyglutamic acid in small peptides was part of the original identification scheme for these residues (Stenflo et al., 1974; Nelsestuen et al., 1974). We have completely decarboxylated the Gla residues of bovine prothrombin and fragment 1 by heating the dried proteins at 110 °C in the absence of moisture and oxygen. By controlling the amount of heating time, we were able to generate a modified protein with any desired level of partial decarboxylation. Recently it was reported that the three Gla residues of calf bone Gla protein could be decarboxylated by a similar method (Poser & Price, 1979), the main procedural difference being the pretreatment of the proteins. Our pretreatment of prothrombin and fragment 1 was chosen with the purpose of retaining the largest possible degree of native conformation.

The protonation state seems to be critical at the dicarboxylic acid side chains of the Gla residues, with the protonated form or the ammonium salt undergoing facile decarboxylation (Poser & Price, 1979). Unlike these authors, who routinely lyophilized their protein from 0.05 M HCl, we lyophilized fragment 1 and prothrombin from water because we found that even traces of HCl caused irreparable damage to the physical structure upon heating. Moreover, below pH 2 prothrombin is irreversibly inactivated in solution with regard to potential thrombin activity. For production of the acidic environment recommended for decarboxylation, our pretreatment included exposure of the protein to acetic acid vapor before evacuation and heating.

The observation that decarboxylation eliminated the specific binding of fragment 1 to barium citrate agrees with the early realization that abnormal prothrombins, later proved to be deficient in Gla, did not bind to barium citrate (Stenflo & Ganrot, 1972; Nelsestuen & Suttie, 1972). In addition, our finding that decarboxylated fragment 1 could still bind to aluminum hydroxide agrees with the report that some abnormal prothrombins not binding to barium salts would bind to alumina (Malhotra, 1972).

Fluorescence intensity spectra and circular dichroic spectra have been presented for normal and fully decarboxylated fragment 1, and both sets were shown to be essentially identical. The conclusion from these data is that the structural integrity of fragment 1 is maintained during the decarboxylation process. The fluorescence experiments have used excitation at 295 nm, thereby observing the intrinsic fluorescence of tryptophan residues only (Prendergast & Mann, 1977). Changes in fluorescence properties are associated with minor local environmental shifts around these tryptophan residues. The CD spectra in the far-ultraviolet spectral region (λ <250 nm) provide information about the overall conformations or secondary structures of proteins in solution. Changes in CD spectra usually reflect more substantial perturbations in the polypeptide architecture. When applied to decarboxylated fragment 1, these spectroscopic techniques did not detect any conformational alterations in solution of either a localized or widespread nature. On the other hand, the fluorescence properties were radically altered under the fully denaturing conditions of 6 M guanidine hydrochloride. Furthermore, the spectroscopic properties of decarboxylated fragment 1 did not show the expected changes in the presence of saturating amounts of Ca^{2+}, specifically fluorescence quenching and CD spectrum transposition. This result confirms the already well established fact that γ-carboxyglutamic acid is necessary for the binding of Ca^{2+} which induces conformational changes as a prelude to prothrombin activation. Recent findings using antibodies directed against the Gla-rich region of prothrombin suggest that this structural transition occurs partly within the Gla-rich region itself (Furie, B., et al., 1978).

Our laboratory has recently proposed a model for prothrombin fragment 1–metal ion interactions (Bloom & Mann, 1978) which argues for the necessity of differential functions among the 10 Gla residues in fragment 1. This model proposes that there are two classes of metal ion binding sites in the fragment 1 region of prothrombin. A high-affinity class of binding sites, two in number (K_d < 0.2 mM for Ca^{2+}), corresponding to Gla-Gla pairs, elicits upon filling a conformational change in the molecule which can be observed by using fluorescence and circular dichroic techniques. A lower affinity class of binding sites, six in number, composed of single Gla residues, is involved in dimerization or binding to phospholipid membranes as hypothesized from kinetic data.

After demonstrating that fragment 1 could successfully be decarboxylated, we proceeded to explore the kinetics of this process by analyzing partially decarboxylated products. The results demonstrate that the rate of loss of the Ca^{2+}-binding function was almost twice as great as the rate of decarboxylation of Gla residues during the initial phase of reaction. This may be explained by the following considerations. The calcium-binding region of the fragment 1 domain of prothrombin contains Gla residues in "pairs" at sequence positions 7/8, 20/21, 26/27, 15/17, and 30/33 (Fernlund et al., 1975; Magnusson et al., 1975). It is reasonable to speculate that two different Gla pairs comprise the two high-affinity Ca^{2+} binding sites as proposed in the foregoing model. Regardless of whether the Gla pairs are adjacent in the sense of either sequence position or spatial juxtaposition, the pairing is deemed functionally significant. In fact, Furie, B. C., et al. (1978) have also proposed that two Gla residues form a high-affinity metal ion binding site based on ^{13}C NMR spectroscopic data. Selective decarboxylation could destroy a Ca^{2+} binding site by removing one carboxyl group from one Gla of a pair; according to this rationale, the decarboxylation of only two or three particular Gla residues would be sufficient to effectively eliminate the calcium-binding capacity. Moreover, since Ca^{2+} binding to fragment 1 is cooperative (Stenflo & Ganrot, 1973; Bajaj et al., 1975), then the removal of one binding site could have an exaggerated effect on the general calcium-binding behavior. This pairing feature and interdependence among Gla residues probably explain why the observed time-dependent decrease in calcium-binding ability is not a simple first-order process.

These results tend to corroborate those presented by Esnouf & Prowse (1977) in a study of some warfarin-induced abnormal prothrombins. A variant human prothrombin which bound to barium citrate showed 68% Gla content compared to normal and 29% biological activity in a Ca^{2+}-dependent venom activation system. Two bovine prothrombins were analyzed: one bound to barium citrate and showed 66% Gla and 4.5% activity, while the other did not bind and showed 40% Gla and <1% activity. A partial chemical analysis of peptides from the fragment 1 region of barium-bound abnormal bovine prothrombin yielded results consistent with the interpretation that one Gla residue from each of the three sequentially adjacent pairs was not carboxylated. Esnouf & Prowse (1977) reached the conclusion that partial noncarboxylation of Gla pairs may exert a disproportionately large effect on the loss of calcium-binding activity.

The nonuniform character of the 10 γ-carboxyglutamic acid residues in fragment 1 is believed to be directly related to the separate functions of Gla within the two classes of metal ion binding sites. It is possible that some Gla residues may be more susceptible than others to decarboxylation depending on their environment in the three-dimensional molecular framework. This is the most likely explanation for the finding that the decarboxylation of fragment 1 is not a first-order process; i.e., all 10 Gla residues may each have a different rate constant for decarboxylation, in which case the observed rate constant would be a complex composite thereof. Fragment 1 is unlike the calf bone Gla protein of Poser & Price (1979) which exhibits first-order decarboxylation kinetics. However, at this point we cannot make the crucial distinction as to whether a partially decarboxylated fragment 1 species is variously decarboxylated at every Gla position or completely modified at only a few Gla positions.

Activation experiments using decarboxylated prothrombin demonstrate that high temperature apparently destroys the

viability of the potential thrombin activity in the COOH-terminal prethrombin 2 domain. This is different from the foregoing abnormal prothrombins which, although they were activated quite slowly in a Ca^{2+}-dependent system, yielded thrombin with the customary specific activity (Prowse et al., 1976). We have been careful to show that there is an introduced defect in the thrombin catalytic process and not just an incomplete conversion of the decarboxylated zymogen to thrombin. Not only has the natural fibrin-related activity been curtailed but also the proteolytic activity toward a small peptide amide substrate. A precedent for this phenomenon has been set: Lundblad et al. (1973) reported that "aged" thrombin incubated for 24 h at 37 °C at pH 7.5 lost 30% of its clotting activity but none of its activity toward Tos-Arg-OMe and showed no evidence of autolysis.

We do not claim that native protein structure is preserved during the decarboxylation process but rather that overall secondary structure is maintained within the limits of spectroscopic detection. Surprisingly little damage occurs with fragment 1. The activity loss accompanying decarboxylation of prothrombin may perhaps be attributed to a localized denaturation or a chemical modification which slightly disturbs the alignment of the catalytic residues in the unexpressed active site of thrombin. Oftentimes, in regard to enzymatic active sites, a subtle structural alteration will have significant functional implications. By contrast, Poser & Price (1979) established in a control experiment that certain enzymes, e.g., chymotrypsin and ribonuclease, could be subjected to the conditions of decarboxylation without losing appreciable enzymatic activity. Thrombin appears to be less stable in this respect.

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