Vitamin K and the Biosynthesis of Prothrombin

II. STRUCTURAL COMPARISON OF NORMAL AND DICOUMAROL-INDUCED BOVINE PROTHROMBIN*

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SUMMARY

Highly purified dicoumarol-induced bovine prothrombin, which does not bind calcium ions and has no prothrombin activity, has been structurally compared with normal prothrombin. Quantitative amino acid and carbohydrate analysis gave identical results for both prothrombins, as did analysis of the NH2-terminal and the COOH-terminal amino acids and molecular weight determination with the sodium dodecyl sulfate gel electrophoretic technique. Peptide maps of tryptic peptides prepared from the reduced and aminoethylated normal and dicoumarol-induced prothrombin were identical. These results suggest that the difference in properties between the two prothrombins are caused by a minor structural difference or a conformational difference.

Ouchterlony immunodiffusion analysis gave a reaction of complete immunological identity between the two prothrombins, whereas the quantitative immunoprecipitation technique indicated antigenic difference between them. Furthermore, it was found that normal prothrombin has calcium ion-dependent antigenic determinants. The sedimentation coefficient, Stokes molecular radius, the titration curves for the tyrosine phenolic groups, and the fluorescence emission spectra were identical, which corroborates that the difference between the normal and the dicoumarol-induced prothrombin does not engage the entire molecule. The results obtained by polyacrylamide gel electrophoresis in 8 M urea may suggest that the difference includes an anomalous pairing of half-cystine residues.

Dicoumarol is an antagonist of vitamin K. Knowledge of the structural difference between this dicoumarol-induced and normal prothrombin is therefore desirable, especially since it might elucidate the role of vitamin K in the bioproduction of extracellular proteins. In this investigation the dicoumarol-induced prothrombin is characterized and its structure is compared with that of normal prothrombin. A preliminary report of parts of this work has been published earlier (2).

EXPERIMENTAL PROCEDURE

Materials

The normal and the dicoumarol-induced prothrombin used in this study were purified in the way described earlier (1). Urea solutions were passed through a mixed bed ion exchange resin prior to use. Guanidine hydrochloride was obtained from Mann ("ultrapure") and used without further purification. Acrylamide and N,N'-methylene bisacrylamide were recrystallized as already described (1). Iodoacetic acid was recrystallized from diethyl ether. Dithiothreitol was obtained from Nutritional Biochemicals, ethyleneimine from Fluka and 5,5'-dithiobis(2-nitrobenzoic acid) from British Drug House Ltd. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, glycine methylester, and anhydrous hydrazine were obtained from Pierce. Phenylisothiocyanate (Eastman), pyridine, and triethylamine were purified as described by Sjöquist (3). Trypsin treated with tosylphenylalanylchloromethyl ketone was obtained from Worthington. Ampholytes (Ampholine) were from LKB Produkter AB, Stockholm, Sweden. Sophora G-100 was obtained from Pharmacia.

Methods

Reduction and Carboxymethylation—Reduction and carboxymethylation was carried out as described by Morino and Snell (4) with the following modifications. The reduction was carried out in 6 M guanidine hydrochloride for 3 hours at 37° and carboxymethylation was allowed to proceed for 2 hours before the sample was dialyzed at 4°, first against 0.1 M NaHCO3 and then thoroughly against distilled deionized water. The precipitated material was lyophilized.

Amino Acid Composition—Lyophilized, salt-free samples of normal and dicoumarol-induced prothrombin (1 to 2 mg) were hydrolyzed in 6 M HCl in sealed, evacuated Pyrex tubes at 110° for 24 and 72 hours (5). The analyses were performed with the two-column system of Spackman et al. (6) on a Jeol model JLC-
5 AH automatic amino acid analyzer. Norleucine was used as an internal standard. Heli-cystine was determined as cysteic acid after performic acid oxidation (7). The tryptophan content was estimated with the technique of Benece and Schmid (8). The amide content was determined with the method of Hoare and Koshland (9): activation of the free carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and reaction with glycine methyl ester resulted in the coupling of glycine to the free carboxyl groups. The reaction was performed with the protein (5 mg) in 5 M guanidine hydrochloride at 25°C in a Radiometer pH-stat with the pH maintained at 4.75 with 0.4 M HCl. The consumption of acid had virtually ceased after 60 min. After extensive dialysis against 1 N HCl and lyophilization the amount of glycine incorporated was determined by amino acid analysis.

The content of free sulfhydryl groups in the two prothrombins was determined with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman (10) in 6 M guanidine hydrochloride in an amount of glycine incorporated was determined by amino acid analysis.

After addition of 500 μl of distilled water the phenylthiohydantoin derivative was performed with 400 μl of HCl-saturated phenylisothiocyanate coupling was performed at 40°C for 2 hours. The pH in the coupling mixture was between 9.5 and 10.0. The reaction was allowed to proceed for 16 hours at 60°C with the NH2 terminal amino acid was determined with the phenylisothiocyanate method of Edman, essentially as described by Jeppsson (11). Prothrombin (6 mg) was dissolved in 300 μl of 0.9% NaCl and mixed with 600 μl pyridine-triethylamine-phenylisothiocyanate (100:3:2, v/v/v). The pH in the coupling mixture was between 9.5 and 10.0. The physisorbed coupling was performed at 40°C for 2 hours. After washing the reaction mixture with 4 × 1 ml of benzene-ethylen chloride-water (3:1:4, v/v/v, upper phase) the sample was taken almost to dryness by evaporation in a vacuum. After addition of 500 μl of distilled water the phenylthiohydantoin amino acids were extracted with 3 × 1 ml of ethylacetate. The combined extracts were taken to dryness by evaporation in vacuo and the residue dissolved in 90% acetic acid. The phenylthiohydantoin amino acids were identified by thin layer chromatography on precoated silica gel plates with fluorescent indicator (Merck). System V of Jeppsson and Sjöquist (12) was used for the chromatography. The spots were eluted with 95% ethanol and the ultraviolet spectra of the phenylthiohydantoin amino acids were recorded.

The COOH-terminal amino acid of normal and dicoumarol-induced prothrombin was determined with hydrazinolysis as described by Frenkel-Conrat and Chunjilng Tsung (13). The reaction was allowed to proceed for 16 hours at 60°C with hydrazine sulfate as a catalyst (14). The dried hydrazinolysate was dissolved in water and the neutral and acidic amino acids were separated from the basic amino acids and from the amino acid hydrazides on a column of Amberlite IRC-50. After lyophilization free amino acids were determined with the amino acid analyzer.

Carbohydrate Analyses—For hexosamine determination the prothrombin was hydrolysed for 6 hours in 4 M HCl at 100°C in sealed, evacuated Pyrex tubes (15). Hexosamine was determined on the short column of the amino acid analyzer with α-amino-β-guanido-propionic acid as internal standard. Standard solutions of glucosamine were subjected to the same treatment. Sialic acid was determined after hydrolysis with 0.1 M H2SO4 at 80°C for 1 hour with the thiobarbituric acid assay (16). Gas chromatographic analysis of the carbohydrates present in one sample of both the normal and the dicoumarol-induced prothrombin was kindly performed by Dr. Hans Bennich at the Wallenberg Laboratory, University of Upsala, using the method of Clamp et al. (17).

Peptide Mapping—Complete reduction and aminooxylation of normal and dicoumarol-induced prothrombin were carried out essentially according to Stobin and Singer (18). A 0.25 to 0.5% solution of the protein in 0.2 M Tris-HCl, 0.01 M EDTA, 7 M guanidine hydrochloride was made 0.1 M in dithiothreitol. The vial was flushed with N2, stopped, and incubated at room temperature. After 2 hours an equal volume of 3 M Tris-HCl, 0.01 M EDTA, 7 M guanidine hydrochloride pH 8.0 was added. With the solution still under N2 ethyleneamine was added in five equal portions at 5-min intervals to a 10-fold molar excess over dithiothreitol. One hour after the first addition of ethyleneamine 1 volume of ice cold, distilled and deionized water was added. The solution was dialyzed against distilled, deionized water and lyophilized.

The lyophilized digest was dissolved in distilled water. About 1.5 mg was applied to a Whatman No. 3MM paper. High voltage electrophoresis was performed at 1.5 kv for 31/2 hours in pyridine-acetic acid-water (100:10:890, v/v/v) at pH 6.1 on an apparatus with a water cooled plate (19). In some experiments a guide strip was stained with ninhydrin to locate the neutral peptides which were cut out and sewn to another Whatman No. 3MM paper.

Electrophoresis was then run at 2.5 kv for 45 min in formic acid-acetic acid-water (10:29:193, v/v/v) at pH 1.8 in a tank under Warsol (20). After electrophoresis the papers were dried by fanning, sewn to new sheets of Whatman No. 3MM paper, and subjected to ascending chromatography in pyridine-isomyl alcohol-water (35:35:30, v/v/v) for 14 to 16 hours (21). The air-dried papers were dipped in 0.1% trichloroacetic acid in acetone and developed at room temperature overnight. Alternatively they were stained for sulfur with platinic iodide followed by a basic ninhydrin stain (22).

Cyanogen Bromide Treatment—Cyanogen bromide degradation was performed with the method described by Steers et al. (20). The protein (5 mg) was dissolved in 90% formic acid. After dilution with water to 70% formic acid cyanogen bromide (250-fold molar excess relative to methionine) was added. The reaction was allowed to proceed for 20 to 24 hours at room temperature, after which the sample was diluted with water (10:1) and the proteins recovered by lyophilization.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing—Polyacrylamide gel electrophoresis in urea was carried out either at pH 8.9 in 8 M urea using 5% or 7% acrylamide gel (24) or at pH 2.7 in 9 M urea using 5% acrylamide gel (25). The electrophoresis was performed at 1.5 ma per tube for about 4 hours. The gels were stained with 0.05 ± Amido black B (Merck) in 7% acetic acid and destained electrophoretically. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as described by Weber and Osborn (26) using 40% of the standard concentration of methyleneoxyacrylamide. Tyrogbolin, phos forylase a, γ-globulin, H chain and horse liver alcohol dehydrogenase were used as molecular weight markers using the molecular weights given by Weber and Osborn (26).
acrylamide gel essentially as described by Awedh et al. (27). The composition of the solutions was identical to that used by Spencer and King (28) except that the urea concentration in the gel was increased to 7.5 M. A mixture of equal volumes of pH 3 to 5 and pH 5 to 7 Ampholine was used with the addition of 10% of this volume of pH 3 to 10 Ampholine. The carbon anode and cathode were wetted with 20% phosphoric acid and 20% ethylenediamine, respectively, immediately prior to the run. Electrophoresis was performed at room temperature for about 16 hours. The voltage was increased slowly to 400 volts so that the current did not exceed 6 mA. The gels were stained in a mixture of 200 ml of 5% trichloroacetic acid-5% sulfosalicylic acid, 2 ml of 1% Coomassie blue and 40 ml of methanol (28).

**Extinction Coefficients**—The concentrations of the purified prothrombins were measured from the absorbance at 280 nm. The extinction coefficients (\(E_{280}^{	ext{nm}}\)) at 280 nm of the purified proteins were determined in the following way. After filtration through a column of Sephadex G-25 fine in 0.05 M phosphate buffer pH 7.5, the protein-containing fractions were pooled and the absorbance at 280 nm measured in a Zeiss PM Q II spectrophotometer. Nitrogen determinations were carried out on the same samples using a micro Kjeldahl technique.1 From these data the extinction coefficients were calculated using the values for nitrogen content (see below) obtained from the amino acid and carbohydrate analyses. One determination was made on each of three different preparations of the two prothrombins. For the normal prothrombin \(E_{280}^{	ext{nm}}\) at 280 nm was 14.6 (14.8, 14.2, 14.9) and for the dicoumarol-induced prothrombin \(E_{280}^{	ext{nm}}\) was 14.4 (13.1, 15.1, 14.9).

**Determination of Sedimentation Coefficient and Stokes Radius**—Ultracentrifugal analyses were performed at 20°C in 0.05 M phosphate buffer, 0.5 M NaCl pH 7.0 in a Spino model E analytical ultracentrifuge equipped with the schlieren optical system. Sedimentation determinations were carried out on the same samples using a micro Kjeldahl technique.1 From these data the extinction coefficients were calculated using the values for nitrogen content (see below) obtained from the amino acid and carbohydrate analyses. One determination was made on each of three different preparations of the two prothrombins. For the normal prothrombin \(E_{280}^{	ext{nm}}\) at 280 nm was 14.6 (14.8, 14.2, 14.9) and for the dicoumarol-induced prothrombin \(E_{280}^{	ext{nm}}\) was 14.4 (13.1, 15.1, 14.9).

**Chemical Composition of Normal and Dicoumarol-induced Prothrombin**

**Amino Acid Composition**—The amino acid composition was determined on two preparations of each of the two prothrombins. As shown in Table I, the compositions of the normal and the dicoumarol-induced prothrombin appeared to be identical. The half-cystine value for the dicoumarol-induced prothrombin is probably one residue too low since titrations with 5,5'-dithiobis(2-nitrobenzoic acid) in 5.5 M guanidine hydrochloride indicated that there was no free sulphydryl group in either of the two prothrombins. This is corroborated by the fact that separate sulfur determinations (36) gave a value of 1.24% for both prothrombins, whereas judging from the amino acid analyses, the sulfur content is 1.22% and 1.17% for the normal and the dicoumarol-induced prothrombin. Separate phosphorus determinations (37) gave values corresponding to less than 1 mole of phosphorus per mole of normal prothrombin and less than 2 moles per mole of dicoumarol-induced prothrombin.6

**End Group Analyses**—Quantitative NH₂-terminal amino acid determinations with a modified Edman procedure (11) repeatedly yielded a single spot on the thin layer chromatograms with a \(R_F\) value corresponding to that of the phenylthiohydantoin of alanine and with a typical ultraviolet spectrum. The recovery was

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1 These analyses were performed at the Centrala Analyslaboratoriet, Department of Chemistry, Uppsala University.

2 The ultracentrifugal analyses were kindly performed by Dr. U. B. Hansson.

3 I wish to thank Professor W. Kirsten, Department of Chemistry, Lantbrukskögskolan, Uppsala, for performing the sulfur and phosphorus analysis.
hydrazides of glycine and serine are known to be very labile, hydrazine had been refluxed on sodium hydroxide, distilled under
and 1.2 residues per 72,000 g of protein if a recovery factor of
released from both prothrombins. The yield varied between 0.65
hydrazine sulfate as catalyst serine was the principal amino acid
protein (uncorrected for operational losses) for both the normal and
0.6 to 0.7 mole of phenylthiohydantoin-alanine per mole of pro-
acid was released in agreement with the results earlier obtained
by Magnusson (38).
Carbohydrate Analysis—The data obtained for the carbohy-
drate analyses of the normal and dicoumarol-induced prothrom-in are summarized in Table II. The values are in fair agree-
ment with those reported earlier by Magnusson (39). There
was no significant difference in the composition of the carbo-
hydrate prosthetic group between the normal and dicoumarol-
duced prothrombin. Glucosamine analyses were performed
both on the short column of the amino acid analyzer and by gas
liquid chromatography. The cause of the discrepancy between
the glucosamine values obtained with the two methods was not
investigated. Sialic acid determinations with the thiobarbituric acid method gave values between 3% and 4% for both prothrom-ins. On agarose gel electrophoresis at pH 8.6 of the purified
normal and dicoumarol-induced prothrombins before and after
digestion with neuraminidase, the electrophoretic mobilities of both proteins were reduced to the same extent, which also indi-
cates an identical number of sialic acid residues.

Peptide Mapping—Tryptic peptide maps were prepared from
the normal and the dicoumarol-induced prothrombin, reduced,
and aminomethylated in 7 M guanidine hydrochloride (Fig. 1).
To improve the separation of the neutral peptides they were cut
out, stitched to another paper, and resolved by a second elec-
rophoretic patterns of normal and dicoumarol-induced pro-
thesis at pH 1.8 followed by ascending chromatography
(Table II)

| Carbohydrate | Normal prothrombin | Dicoumarol-induced prothrombin |
|--------------|--------------------|--------------------------------|
| (N-Acetyl) glucosamine | 4.5 | 5.1 |
| Mannose | 2.4 | 2.5 |
| Galactose | 3.3 | 3.0 |
| Sialic acid | 3.4 | 3.9 |

Table I
Amino acid composition of normal and dicoumarol-
induced prothrombin

The values represent the average from one 24- and one 72-hour
hydrolysis on each of two different preparations of both pro-

| Amino acid | Normal prothrombin | Dicoumarol-induced prothrombin |
|------------|-------------------|--------------------------------|
| Lysine | 31.2 | 33.0 |
| Histidine | 9.9 | 10.1 |
| Arginine | 40.0 | 38.2 |
| Half-cystine | 19.8 | 18.8 |
| Aspartic acid | 56.3 | 56.7 |
| Threonine | 27.8 | 28.4 |
| Serine | 37.8 | 37.3 |
| Glutamic acid | 68.9 | 70.1 |
| Proline | 34.0 | 34.6 |
| Glycine | 44.4 | 44.6 |
| Alanine | 31.8 | 34.1 |
| Valine | 33.0 | 34.5 |
| Methionine | 7.7 | 7.4 |
| Isoleucine | 16.5 | 19.4 |
| Leucine | 43.0 | 42.7 |
| Tyrosine | 17.1 | 16.0 |
| Phenylalanine | 19.5 | 18.7 |
| Tryptophan | 15.0 | 14.2 |
| Asparagine plus glutamine | 52.0 | 50.2 |

a The values are calculated for a molecular weight of 72,000 for
both prothrombins and assuming a carbohydrate content of 12.5%.
b Determined separately after performic acid oxidation.
c Extrapolated to zero time of hydrolysis.
d Values taken from the 72 hours of hydrolysis time.
e Measured spectrophotometrically."f Measured as described in the text. Values obtained from one
determination on each of the two prothrombins.

0.6 to 0.7 mole of phenylthiohydantoin-alanine per mole of pro-
tein (uncorrected for operational losses) for both the normal and
the dicoumarol-induced prothrombin.

On hydroxylation with anhydrous hydrazine (Pierce) and with
hydrazine sulfate as catalyst serine was the principal amino acid
released from both prothrombins. The yield varied between 0.65
and 1.2 residues per 72,000 g of protein if a recovery factor of
96% is anticipated (14). Glycine was regularly demonstrated in
amounts of 0.2 to 0.3 residue per mole of protein, whereas alanine,
aspartic acid, and threonine varied between 0.1 and 0.2 mole per
mole of protein. Identical results were obtained when the
hydrazine had been refluxed on sodium hydroxide, distilled under
nitrogen, and used immediately. If unsatisfactory hydrazine
was used the amount of glycine increased considerably, whereas
the amount of serine released was fairly constant. Since the
hydrazines of glycine and serine are known to be very labile,
control experiments on human transferrin and bovine serum
albumin were performed. The amount of serine released in these
proteins was negligible. Attempts were made to check that
serine is the carboxy terminal amino acid by carboxypeptidase
A and B digestion of reduced and carbamylmethylated samples in
0.2 M Tris-HCl buffer, pH 8.0, 0.05 M in sodium lauryl sulfate
(13). Samples removed after various time intervals were ana-
lyzed on the automatic amino acid analyzer. However, no amino

Properties of Normal and Dicoumarol-induced Prothrombin
Polyacrylamide Gel Electrophoresis—The polyacrylamide gel
electrophoretic patterns of normal and dicoumarol-induced pro-

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thrombin at pH 8.9, 8 M urea and at pH 2.7, 9 M urea are shown in Fig. 3. Mixtures of reduced and alkylated normal and dicoumarol-induced prothrombin migrated as a single band at both pH values, whereas mixtures of the two unmodified prothrombins could be completely separated into two protein bands in prolonged electrophoretic runs with the normal prothrombin in the most anodal position. At pH 2.7 no separation could be achieved between unmodified normal and dicoumarol-induced prothrombin. At present no entirely satisfactory explanation can be given for the fact that the two untreated prothrombins separated at pH 8.9, but not at pH 2.7. However, it was probably not due to charge differences between the two proteins, but possibly to more complete denaturation, and thus a larger molecular radius of the abnormal prothrombin than of the normal prothrombin in the slightly alkaline pH range. Since urea is a more efficient denaturing agent at low pH values (40), both prothrombins are probably completely denatured at pH 2.7 and consequently might have the same molecular radius and electrophoretic mobility.

To confirm that there is no charge difference between the two prothrombins they were analyzed by isoelectric focusing in polyacrylamide gel containing 7.5 M urea in a pH 3 to 7 gradient. Three bands were regularly seen in both prothrombins. However, the normal and the dicoumarol-induced prothrombins gave identical patterns (Fig. 4).

**Fig. 4.** Isoelectric focusing in a pH 3 to 7 gradient in polyacrylamide gel containing 7.5 M urea. Dicoumarol-induced prothrombin (A) and normal prothrombin (B).

Molecular Size of Normal and Dicoumarol-induced Prothrombin

—Sedimentation velocity studies of the normal and the dicoumarol-induced prothrombin in 0.05 M phosphate buffer (pH 7.0), 0.5 M NaCl in the wedge cell revealed two single symmetrical peaks sedimenting with apparently the same velocity (Fig. 5). The value for the $s_{20,w}$ of the dicoumarol-induced prothrombin extrapolated from sedimentation runs at four different protein concentrations was 4.9.

The Stokes molecular radius for the two prothrombins was determined on a calibrated column of Sephadex G-100. A buffer with high ionic strength was chosen since at lower ionic strengths Tishkoff et al. (41) have shown that the elution volume is mark-
FIG. 5 (upper). Schlieren patterns obtained in the wedge cell of purified dicoumarol-induced prothrombin (upper) and normal prothrombin (lower). The proteins had been previously dialyzed against 0.05 M phosphate buffer 0.5 M NaCl, pH 7.0. The pictures were taken 64, 80, and 112 min after reaching a speed of 59,780 rpm. Protein concentration 9.0 mg per ml.

FIG. 6 (lower). Polyacrylamide gel electrophoretic pattern in sodium dodecyl sulfate of mixture of normal and dicoumarol-induced prothrombin. The arrow indicates the origin. Forty per cent of the amount, of bisacrylamide employed in the standard procedure was used.

FIG. 7. Ouchterlony immunodiffusion analyses of normal (A) and dicoumarol-induced prothrombin (B) with an antiserum raised against normal bovine prothrombin.

edly dependent on the protein concentration in a way that suggests that the prothrombin molecules undergo reversible associations. Duplicate determinations on the same column gave values between 40 and 41 Å for both prothrombins.

To obtain evidence for the assumed identity in molecular weight for the two prothrombins electrophoresis in sodium dodecyl sulfate was carried out according to Weber and Osborn (26). Mixtures of normal and dicoumarol-induced prothrombin gave only one protein band (Fig. 6). A molecular weight of 72,000 ± 1,000 (average of three determinations with four molecular weight markers) was obtained.

Immunological Properties—When the normal and the dicoumarol-induced prothrombins were analyzed by crossed immunoelectrophoresis in calcium ion containing buffer, the precipitate produced by the normal prothrombin was heavier stained than the precipitate produced by the dicoumarol-induced prothrombin both when unfractionated plasma samples and mixtures of the two purified proteins were analyzed (1). This finding prompted an investigation of the two proteins with the Ouchterlony immunodiffusion technique. The result obtained with an antiserum raised against normal bovine prothrombin was compatible with complete identity of the two prothrombins (Fig. 7).

To obtain more detailed information the quantitative precipitin technique was used. Precipitation curves were prepared from both prothrombins with buffer and antisera containing either 5 mM EDTA or 5 mM CaCl₂ (Fig. 8). With the dicoumarol-induced prothrombin there was only a slight decrease in precipitation with EDTA instead of Ca²⁺, presumably due to decomplementation of the antisemur (42). In contrast with these findings, the normal prothrombin exhibited far greater precipitation in the equivalence zone in the presence of Ca²⁺ than in the presence of EDTA. The precipitation was also greater than that obtained with the dicoumarol-induced prothrombin. These results indicate a Ca²⁺-induced conformational change in the normal prothrombin. Furthermore the dicoumarol-induced prothrombin has a conformation different from that of normal prothrombin both with and without Ca²⁺.

Other Properties of Normal and Dicoumarol-induced Prothrombin—The difference between the two prothrombins in calcium ion binding, electrophoretic mobility, and immunochemical properties indicated that they had different conformations. Attempts were therefore made to corroborate this by spectrophotometric titration of tyrosine residues and by measuring fluorescence emission spectra. However, the tyrosine titration curves proved identical for both prothrombins. The titration data indicated 18 tyrosine residues, approximately 5 of which were freely exposed to titration, whereas the remaining ones seemed to have anomalously high pK values. Ca²⁺ or 5 mM EDTA in the buffer did not influence the shape of the titration curves. Fluorescence emission spectra were recorded with excitation at 280 nm. The spectra of the normal and the dicoumarol-induced prothrombin were identical with maxima at 340 nm. Emission spectra recorded after excitation at several
different wave lengths did not reveal any nonprotein chromophores. Both normal and dicoumarol-induced prothrombin contain 7 to 8 methionine residues. On polyacrylamide gel electrophoretic analyses in 8 M urea of cyanogen bromide fragments from the two prothrombins with intact disulfide bonds two major protein zones were visible at pH 8.9 and four at pH 2.7. The protein zones obtained with the two prothrombins seemed identical at both pH values. However, when mixtures of cyanogen bromide fragments from the normal and the dicoumarol-induced prothrombin were analyzed on the same gel, a pattern with four protein zones was repeatedly obtained indicating small differences in electrophoretic mobility between the fragments from the two prothrombins at pH 8.9. At pH 2.7 the mixture gave a pattern identical with that obtained with the individual proteins (Fig. 9).

DISCUSSION

The preparations of normal and dicoumarol-induced prothrombin used in the present study are homogenous by several criteria including electrophoresis in agarose gel and polyacrylamide gel without dissociating medium (1) and with 8 or 9 M urea at two different pH values. Yet molecular polymorphism was found in purified preparations of the two prothrombins when analyzed by isoelectric focusing in polyacrylamide gel containing 7.5 M urea. Three fractions were found in both the normal and in the dicoumarol-induced prothrombin. There was no difference between the two proteins. Since the purification methods used for the two prothrombins are entirely different (1) this microheterogeneity is probably not a preparation artifact.

The abnormal prothrombin synthesized during dicoumarol administration as well as the normal prothrombin has a molecular weight of 72,000 as determined with the sodium dodecyl sulfate gel electrophoresis technique, which is in agreement with the sedimentation equilibrium molecular weight of 74,000 reported by Ingwall and Scheraga (43) for normal prothrombin. The results of amino acid and carbohydrate analysis reported in this paper are in agreement with values reported earlier (44) and identical for the normal and the dicoumarol-induced prothrombin. In both, 1 residue of alanine per molecule of prothrombin was found as amino terminus in agreement with several earlier reports (45-47). Attempts to determine the COOH-terminal amino acid with the carboxypeptidase method were not successful, as in Magnusson's (38) investigation. Therefore, hydrazinolysis was resorted to. With this method a reasonable yield of serine was obtained from both prothrombins. This is in agreement with the proposal of Magnusson (48) that the β chain of thrombin which has a COOH-terminal serine constitutes the COOH-terminal portion of the prothrombin molecule. However, definite conclusions regarding the COOH-terminal amino acid of prothrombin must await confirmation with other methods.

Several analyses of tryptic peptide maps of the normal and the dicoumarol-induced prothrombin gave very reproducible results. That the number of peptides predicted from the amino acid composition was not obtained might be due to some homologous amino acid sequences in the thrombin and the non-thrombin parts of the molecule. This explanation is supported by the finding that on activation prothrombin gives rise not only to one enzyme with esterase activity, i.e. thrombin, and that the non-thrombin part of the molecule also exhibits esterase activity (49).

Peptide maps of both the normal and the dicoumarol-induced prothrombin were identical with respect to all the major peptide spots. The results hitherto presented indicate that the differences in properties between the two prothrombins are caused by minor structural differences, e.g. in disulfide pairing or in prosthetic group, or by conformational differences.

The finding that the two prothrombins have the same main antigenic determinants and that the sedimentation coefficients, the Stokes molecular radius, the tyrosine titration curves, and the fluorescence emission spectra were identical indicates that there is no gross difference involving the entire molecule. The quantitative precipitin curves furthermore suggest that there is a conformational difference between normal prothrombin with and without calcium ions which is in agreement with the observation that the autoactivation of prothrombin is enhanced by calcium ions. No such calcium ion-dependent conformation was observed in the dicoumarol-induced prothrombin. Similar calcium-dependent antigenic determinants have recently been identified with the quantitative precipitin technique in glutamic acid containing synthetic polypeptide acids such as (L-Glu-Ala-L-Tyr)₉₀ by Maurer et al. (50).

The differences in electrophoretic mobility of the two prothrombins in 8 M urea at pH 8.9 when the disulfide bonds are intact seems to rule out a differential noncovalent binding of small ligands as a cause of the difference in calcium ion binding and immunochromatographic properties between the two proteins. The cyanogen bromide degradation of the two prothrombins with intact disulfide bonds resulted in fragments with slightly different mobility at pH 8.9, in agreement with the results obtained with the intact proteins. The unsuccessful attempts to distinguish between the two prothrombins when they are reduced and alkylated, whereas electrophoretic separation could be achieved by polyacrylamide gel electrophoresis in 8 M urea when the disulfide bonds are intact may be compatible with the idea that there is a different pairing of disulfide bonds in the two prothrombins.
Since the concept of stable conformational variants might have a bearing on the present results attempts to reoxidize the normal prothrombin from random coil have been undertaken as suggested by Epstein and Schechter (31). However, so far such attempts have yielded only high molecular weight prothrombin aggregates despite a wide variation of the reoxidation conditions with regard to pH, protein concentration, mercaptoethanol concentration, and calcium ion concentration. Similarly, attempts to normalize the abnormal prothrombin by incubation with mercaptoethanol at a physiological pH have been unsuccessful.

Using the immunofluorescence technique Burnbart and Anderson (52) could not identify any precursor prothrombin in the liver of dogs with the same antigenic determinants as prothrombin. However, in the rat it has been suggested that a precursor of prothrombin is accumulated in the liver in vitamin K deficiency which upon administration of the vitamin is completed and released to the circulation (53-56). This has also been suggested by Shah and Suttie (57) who demonstrated that the prothrombin formed in vitamin K deficient rats when radioactive amino acids and the vitamin are administered after cyclohexamide administration contains no radioactivity. If the dicoumarol-induced prothrombin has any relation to a prothrombin precursor cannot be evaluated at present.

The fact that the normal and the dicoumarol-induced prothrombin are identical in carbohydrate composition and have identical peptide maps is incompatible with the recent suggestion that dicoumarol adversely affects the carbohydrate attachment to the polypeptide chain (58, 59). Furthermore, the demonstration that the abnormal prothrombin does not possess prothrombin activity (1) is not in accord with this hypothesis, since it has been demonstrated that removal of a substantial amount of the carbohydrate from normal prothrombin has no effect on the biological activity of the protein or on its adsorption to barium salts (60). Further experimental work on the covalent structure and the conformation of the normal and the dicoumarol-induced prothrombin is necessary before any hypothesis on the mode of action of vitamin K can be arrived at. However, the fact that vitamin K can function as an oxidation-reduction mediator in the cell and that dicoumarol inhibits the enzyme vitamin K reductase (61) or DT diaphorase (EC 1,6.99.2) (62) makes it tempting to speculate that there is an abnormal pairing of disulfide bridges in the dicoumarol-induced prothrombin which is also consistent with the experimental results so far obtained.

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