Vitamin K and the Biosynthesis of Prothrombin

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

JOHAN STENFLO

From the Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden

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 is 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 biosynthesis 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 PROCEUURE

Materials

- The normal and the dicoumarol-induced prothrombin used in this study were purified in the way described earlier (1). Urea solutions were treated 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 diethylether. Dithiothreitol was obtained from Nutritional Biochemicals, ethyleneimine from Fluka and 5,5'-dithiobis(2-nitrobenzoic acid) from British Drug House Ltd. L-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. Sephadex G-100 was obtained from Pharmacia.

Methods

- Reduction and Carboxymethylation—Reduction and carboxymethylation was carried out as described by Merino 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 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 N 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. Half-cystine was determined as cysteic acid after performic acid oxidation (7). The tryptophan content was estimated with the technique of Bence 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 methylester resulted in the coupling of glycine to the free carboxyl groups. The reaction was performed with the protein (5 mg) in 5 ml 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 M 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 0.6 M guanidine hydrochloride in 0.1 M Tris-HCl, 5 M EDTA, pH 7.5.

End Group Analysis—The NH₂-terminal amino acid was determined with the phenylsihthiocyanate method of Edman, essentially as described by Jeppson (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 phenylisothiocyanate coupling was performed at 40°C for 2 hours. After washing the reaction mixture with 4 × 1 ml of benzene-ethylene chloride-water (3:1:4, v/v/v, upper phase) the sample was dried by fanning, sewn to new sheets of Whatman No. 3MM paper, and developed at room temperature overnight. Alternatively the spots were stained for sulfur with platinic iodide followed by a basic ninhydrin stain (22).

The COOH-terminal amino acid of normal and dicoumarol-induced prothrombin was kindly performed by Dr. Hans Bennich at the Wallenberg Laboratory, University of Uppsala, 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 Slobin 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 diithiothreitol. 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 ethyleneimine was added in five equal portions at 5-min intervals to a 10-fold molar excess over diithiothreitol. One hour after the first addition of ethyleneimine 1 volume of ice cold, distilled and deionized water was added. The solution was dialyzed against distilled, deionized water and lyophilized.

The reduced, aminooxylated protein was suspended to a concentration of 5 mg per ml in 0.1 M NH₄HCO₃ pH 8.5 containing phenol red as a pH indicator. Digestion was carried out with tosylphenylalaninechloromethyl ketone-treated trypsin at 37°C for 8 to 14 hours at an enzyme to substrate ratio of 1:50 (w/w). At the end of the digestion when no core remained, the samples were frozen 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 3½ 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 Worsal (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-isoamyl alcohol-water (35:35:30) v/v/v for 14 to 16 hours. The air-dried papers were dipped in 0.5% ninhydrin in acetone and developed at room temperature overnight. Alternatively they were stained for sulfur with platlnic iodide followed by a basic ninhydrin stain (22).

Cyanogen Bromide Treatment—Cyanogen bromide degradation was performed with the method described by Stigers et al. (22). 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 8.0 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 (Merek) 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 methylendibacrylamide. Thyroglobulin, phos phorylase, α-globulin, H chain and horse liver alcohol dehydrogenase were used as molecular weight markers using the molecular weights given by Weber and Osborn (26).

Isoelectric focusing was carried out in thin layers of poly-
acylamide 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% ethylendiamine, respectively, immediately prior to the run. Electrofocusing 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 \(E_{280}^m\) 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. 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 \(E_{280}^m\) at 280 nm was 14.6 (14.8, 14.2, 14.9) and for the dicoumarol-induced prothrombin \(E_{280}^m\) was 14.4 (13.1, 15.1, 14.9).

**Determination of Sedimentation Coefficient and Stokes Radii**—Ultracentrifugal analyses\(^2\) were performed at 20\(^\circ\)C in 0.05 M phosphate buffer, 0.5 M NaCl in SaCl pH 7.0 in a Spinco model E analytical ultracentrifuge equipped with the schlieren optical system. Sedimentation runs were made at 60,780 rpm in 12 mm 4\(^\prime\) single sector cells. A direct comparison between normal and dicoumarol-induced prothrombin was performed in wedge cells. All calculations were corrected to water at 20\(^\circ\)C and zero protein concentration as described by Schachmann (29).

Stokes radius was determined with the method of Laurent and Killander (30). Analytical gel chromatography was carried out at 4\(^\circ\)C on a column (1.20 \times 104 cm) of Sephadex G-100 equilibrated with 0.04 M Tris-HCl, 0.5 M NaCl and 2 mM EDTA pH 7.4. The column was equipped with flow adapters and eluted upward at a flow rate of 2.9 ml per cm\(^2\) per hour with a Persepolis peristaltic pump (LKB, Stockholm, Sweden). The column effluent was continuously monitored at 280 nm with a Uvicord at a flow rate of 2.9 ml per cm\(^2\) per hour with a Persplex sector cells. B direct comparison between normal and dicoumarol-induced prothrombin was performed in wedge cells. All calculations were corrected to water at 20\(^\circ\)C and zero protein concentration as described by Schachmann (29).

Stokes radius was determined with the method of Laurent and Killander (30). Analytical gel chromatography was carried out at 4\(^\circ\)C on a column (1.20 \times 104 cm) of Sephadex G-100 equilibrated with 0.04 M Tris-HCl, 0.5 M NaCl and 2 mM EDTA pH 7.4. The column was equipped with flow adapters and eluted upward at a flow rate of 2.9 ml per cm\(^2\) per hour with a Persepolis peristaltic pump (LKB, Stockholm, Sweden). The column effluent was continuously monitored at 280 nm with a Uvicord (LKB, Stockholm, Sweden). All analyses were performed in duplicate. \(K_{sv}\) was measured as described by Karlsson et al. (31). Stokes molecular radius was calculated from the formula given by Laurent and Killander (30).

\[
K_{sv} = \frac{r_s^2}{L(L + r_s)^4}
\]

where \(K_{sv}\) is the volume available for the protein in the gel, \(r_s\) is Stokes radius, \(L\) and \(r_s\) are constants characteristic of the gel. \(L\) was determined from the \(K_{sv}\) of human albumin \(r_s = 35.5\) A and \(r_s\) was assumed to be 6.5 A (30, 32).

**Immunological Methods**—Double immunodiffusion was carried out as described by Ouchterlony (33). Quantitative precipitation of normal and dicoumarol-induced prothrombin was performed with an antiserum raised against normal bovine prothrombin (1). The antiserum was diluted 1:3 and dialyzed against 0.05 M Tris-HCl buffer, 5 mM in EDTA, pH 7.4. Various amounts of the antigens dialyzed against the same buffer were added to 0.5 ml of the antiserum and the final volumes made up to 1.0 ml with the buffer. The tubes were incubated at 37\(^\circ\)C for 1 hour and then at 4\(^\circ\)C for 16 hours. The precipitates were collected by centrifugation, washed twice with the above buffer, and then dried. One-tenth milliliter of 0.1 M NaOH was added and the samples warmed until the precipitates dissolved, after which 1.2 ml of 6 M guanidine hydrochloride in 0.4 M Tris-HCl buffer, 5 mM in EDTA pH 7.4 was added. After careful mixing the absorbances at 280 nm were recorded. In some experiments the same Tris buffers were used, but containing 5 mM calcium chloride instead of EDTA.

**Spectrophotometric Titration of Tyrosine Phenolic Groups**—The two prothrombins were dialyzed against 0.05 M Tris-HCl, 0.15 M NaCl pH 8.0. Titrations were made in 1-cm quartz cells in a Zeiss PMQ II spectrophotometer using protein concentrations of 0.4 to 0.5 mg per ml. Small additions of NaOH were made with a Hamilton microliter syringe followed by thorough mixing. After each addition the pH of the sample was measured and the absorbance at 280 nm, recorded. The absorbance values were corrected for the small changes in volume. The molar absorbance increase at 280 nm, was assumed to be 2100 per phenolic group titrated (34, 35).

**Fluorescence Spectra**—Fluorescence emission spectra were measured at ambient temperature (23 ± 1\(^\circ\)) in 1-cm cells in an Amino-Bowman spectrophotofluorometer equipped with an X-Y recorder (Houston Omnigraph 2000). The excitation and emission slit widths were 1.0 and 0.5 mm, respectively. Measurements were made with protein concentrations of 0.05 mg per ml in 0.05 M Tris-HCl buffer pH 8.0 which was 0.15 M in NaCl and 5 mM in EDTA. Only uncorrected spectra were recorded.

**RESULTS**

**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'-dithio-bis(2-nitrobenzoic acid) in 5.5 M guanidine hydrochloride indicated that there was no free sulfhydryl 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.

**End Group Analyses**—Quantitative NH\(_2\)-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 phenylthiobhydantoin of alanine and with a typical ultraviolet spectrum. The recovery was...
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 prothrombins.

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

* The values are calculated for a molecular weight of 72,000 for both prothrombins and assuming a carbohydrate content of 12.5%.
* Determined separately after performic acid oxidation.
* Extrapolated to zero time of hydrolysis.
* Values taken from the 72 hours of hydrolysis time.
* Determined spectrophotometrically.
* 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 protein (uncorrected for operational losses) for both the normal and the dicoumarol-induced prothrombin.

On hydrazinolysis 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 hydrazides 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 carbonyl terminal amino acid by carboxypeptidase A and B digestion of reduced and carboxymethylated 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 analyzed on the automatic amino acid analyzer. However, no amino acid was released in agreement with the results earlier obtained by Magnusson (38).

Carbohydrate Analysis—The data obtained for the carbohydrate analyses of the normal and dicoumarol-induced prothrombin are summarized in Table II. The values are in fair agreement with those reported earlier by Magnusson (39). There was no significant difference in the composition of the carbohydrate prosthetic group between the normal and dicoumarol-induced 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 prothrombins. 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 indicates an identical number of sialic acid residues.

Peptide Mapping—Tryptic peptide maps were prepared from the normal and the dicoumarol-induced prothrombin, reduced, and aminoethylated in 7 M guanidine hydrochloride (Fig. 1). To improve the separation of the neutral peptides they were cut out, stained somewhat heavier in the map of the dicoumarol-induced prothrombin. Heating of the peptide maps at 80°C gave no additional spots. Twenty-four of the spots were positive in the platinic iodide stain, whereas 27 to 28 spots were expected from the amino acid composition. The peptide spot marked 1 (Fig. 1) is stained somewhat heavier in the map of the dicoumarol-induced prothrombin but is also present in the map of the normal prothrombin. The peptide spot marked 2, which was positive in the platinic iodide stain, exhibited a somen-hat varying value from one run to another in peptide maps from both prothrombins. Peptide maps prepared from different preparations of the two prothrombins yielded reproducible patterns. Furthermore no core was visible on completion of the tryptic digestion or at the point of application of the digested sample on the paper. Thus, judging from these findings the two prothrombins are identical in amino acid sequence and carbohydrate composition.

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

Downloaded from http://www.jbc.org/ on August 15, 2017
FIG. 1 (left). Comparison of tryptic peptide maps of normal (A) and dicoumarol-induced prothrombin (B). Electrophoresis at pH 6.1 followed by ascending chromatography. For details see "Methods."

FIG. 2 (right). Comparison of peptide maps of neutral peptides from normal prothrombin (A) and dicoumarol-induced prothrombin (B). Electrophoresis at pH 1.8 followed by ascending chromatography. For details see "Methods."

FIG. 3. Polyacrylamide gel electrophoresis at pH 8.9, 8 M urea (A and B) and at pH 2.7, 9 M urea (C and D). All gels contain a mixture of approximately equal amounts of normal and dicoumarol-induced prothrombin, "untreated" (A and C) and reduced and carboxymethylated (B and D).

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).

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).

**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 Tischkoff 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.

Immunoechemical 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 decomposition of the antiserum (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 immunoechemical 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 disassociating 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 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 any minor structural difference, 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-Glu4, L-Ala3, L-Tyr3)9 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 immunochemical 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 result 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 (51). 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.4

Using the immunofluorescence technique Burnhart 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 Sutie (57) who demonstrated that the prothrombin formed in vitamin K deficient rats when radioactively labeled active amino acids and the vitamin are administered after cycloheximide 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 covariant 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 disulphide bridges in the dicoumarol-induced prothrombin which is also consistent with the experimental results so far obtained.

Acknowledgment—The expert technical assistance of Mrs. Monica Jönsson is gratefully acknowledged.

REFERENCES

7. Cohn, J. (1963) J. Biol. Chem. 238, 335

*J. Stenflo, unpublished results.
Vitamin K and the Biosynthesis of Prothrombin: II. STRUCTURAL COMPARISON OF NORMAL AND DICOUMAROL-INDUCED BOVINE PROTHROMBIN

Johan Stenflo


Access the most updated version of this article at http://www.jbc.org/content/247/24/8167

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/24/8167.full.html#ref-list-1