Antibodies specific for bovine abnormal (Des-γ-carboxy-prothrombin)*

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Antibodies specific for abnormal prothrombin (des-γ-carboxy-prothrombin) lacking γ-carboxyglutamic acid were purified from antiabnormal prothrombin antisera. Abnormal prothrombin was purified 550-fold in 30% yield from the plasma of a calf treated with sodium warfarin. The purification procedure included barium citrate absorption, affinity chromatography with prethrombin 1 antibodies covalently bound to Sepharose, and Sephacryl S200 gel filtration. Antibodies to abnormal prothrombin were raised in rabbits. These antibodies formed precipitating complexes with abnormal prothrombin and prothrombin. However, these antibodies cross-reacted with lower affinity with prothrombin fragment 1, prothrombin fragment 12-44, Factor X, and Factor IX, as evaluated using a radioimmunoassay. Antibodies specific for abnormal prothrombin were isolated from antiabnormal prothrombin antisera by sequential immunoabsorption using affinity chromatography. These antibodies were purified using Sepharose covalently linked to des-γ-carboxy-fragment 1 and Sepharose covalently linked to prothrombin. The purified antibody subpopulation was found by radioimmunoassay to bind to abnormal prothrombin but did not cross-react with prothrombin. Prothrombin in 1000-fold molar excess of abnormal prothrombin did not inhibit the interaction of these specific antibodies with abnormal prothrombin. The interaction of these antibodies with abnormal prothrombin was not affected by the presence of CaCl2. The interaction of these antibodies with 125I-labeled abnormal prothrombin was inhibited by the plasma obtained from a calf treated with warfarin but was not significantly inhibited by normal bovine plasma, human plasma obtained from a patient treated with warfarin, or normal human plasma. These studies indicate that human and bovine abnormal prothrombin do not share antigenic determinants against which these antibodies are directed. Furthermore, abnormal prothrombin is not a significant component of normal bovine plasma.

Abnormal prothrombin, or des-γ-carboxy-prothrombin, is a form of prothrombin that circulates in the blood of mammals that are vitamin K-deficient or that have ingested vitamin K antagonists (1-4). This form appears identical with native prothrombin except that it lacks γ-carboxyglutamic acid residues (5, 6). As a consequence of this structural difference, abnormal prothrombin does not bind Ca(II) (2, 7, 8) or lipid micelles (9) and is not converted to thrombin by activated Factor X in the presence of Ca(II), Factor V, and membrane surfaces (4). However, abnormal prothrombin is converted to thrombin by a protease in Echis carinatus venom that does not require Ca(II) as a cofactor (4).

Antibodies to prothrombin cross-react with abnormal prothrombin and, indeed, were fundamental in establishing the presence of abnormal prothrombin in the blood of patients treated with sodium warfarin (1, 2). Although the interaction of anti-prothrombin antibodies with prothrombin is, in part, metal-dependent (10-12), the interaction of antiprothrombin antibodies with abnormal prothrombin is not affected by metal ions (10). An antiprothrombin antibody subpopulation directed against the γ-carboxyglutamic acid-rich region of prothrombin cross-reacts minimally with abnormal prothrombin, indicating that antigenic determinants in the region 12-44 in prothrombin are not shared by abnormal prothrombin (11).

In the current investigation, we have extended these studies by preparing antibodies to abnormal prothrombin. We describe the isolation and characterization of an antiabnormal prothrombin antibody subpopulation which binds to abnormal prothrombin but not to prothrombin.

MATERIALS AND METHODS

Bovine prothrombin, fragment 1, and prothrombin fragment 12-44 were prepared as described previously (11, 13). E\text{360} values of 14.4, 10.5, and 13.6 were employed for prothrombin, fragment 1 and fragment 12-44, respectively. Factor X was purified from citrated bovine plasma by barium citrate absorption and elution, DEAE-Sephadex chromatography and heparin-agarose chromatography (17). The protein was further purified by preparative polyacrylamide gel electrophoresis (18). The initial blood collection, plasma separation, and barium citrate absorption were performed by the New England Enzyme Center (Tufts University School of Medicine). All proteins appeared greater than 98% pure as evaluated by dodecyl sulfate-electrophoresis (19).

Des-γ-carboxy-fragment 1 was prepared by decarboxylation of prothrombin fragment 1 using the procedure of Mann, a method similar to that reported elsewhere (20). High molecular weight protein aggregates formed during decarboxylation were removed from des-γ-carboxy-fragment 1 by gel filtration on Sephacryl S200. The isolated

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1 K. G. Mann, personal communication.
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γ-carboxy-fragment 1 yielded a single band by dodecyl sulfate electrophoresis. On the average, this preparation contained between 1 and 2 mol of γ-carboxyglutamic acid residues/mol of protein.

Goat anti-rabbit immunoglobulin (Cappel Laboratories), bovine albumin (Sigma), and rabbit immunoglobulin (Cappel Laboratories) were obtained commercially. Sepharose 4B, Sephacryl S200, and Dithiothreitol were from Pharmacia-LKB Biotechnology Inc. From the stock solutions, 85 cm of 4 M guanidine HCl and incomplete adjuvant were purchased from Difco. Chloramine-T, guanidine HCl, Tween 20, and benzamidine-HCl were supplied by Eastman, Schwarz/Mann, Fisher, and Aldrich, respectively. 

Preparation of Agarose Derivatives for Affinity Chromatography—Sepharose 4B was activated with cyanogen bromide (Eastman; 1 mg/ml, 24 hr) to yield a single band by dodecyl sulfate electrophoresis. On the average, this preparation contained between 1 and 2 mol of γ-carboxyglutamic acid residues/mol of protein.

Preparation of Abnormal Prothrombin—Bovine abnormal prothrombin was labeled with 125I using chloramine-T (24). NaCl (New England Nuclear: specific radioactivity, 17 Ci/mg) was added to abnormal prothrombin, 50 μg in 100 μl of 0.5 M sodium phosphate, pH 7.5. Chloramine-T (10 μl of 2.5 mg/ml) was added to the solution and the reaction was stopped at 45 s with the addition of 20 μl of sodium bisulfite, 2.5 mg/ml. After the addition of bovine albumin to a final concentration of 0.05% (w/v) to minimize protein leakage from the column, the labeled abnormal prothrombin was exhaustively dialyzed against 40 mM Tris-HCl (pH 8.1), 0.15 M NaCl. 125I-labeled prothrombin was prepared by the same method.

Preparation of Antibody—New Zealand white rabbits were immunized with either bovine prothrombin or bovine abnormal prothrombin. Prothrombin or abnormal prothrombin was dialyzed at 4°C against 40 mM Tris-HCl (pH 8.1), 0.15 M NaCl. The protein solution (1 mg/ml) was emulsified in 1 ml of Freund's adjuvant. Rabbits were injected subcutaneously with the emulsion in multiple sites every 2 weeks for 2 months using Freund's complete adjuvant and then injected monthly using Freund's incomplete adjuvant. Antibodies were collected from the lateral ear vein and the antisera were pooled, collected, and stored at −15°C. Antibody concentration was estimated by quantitative precipitin analysis.

Isolation of Abnormal Prothrombin-specific Antibodies—Antibodies specific for abnormal prothrombin were purified by sequential immunabsorption employing affinity chromatography. Antinormal prothrombin antisera (5 ml) was applied to a Sepharose-des-γ-carboxy-fragment 1 column (1.0 × 4.5 cm) at 23°C equilibrated with BBS/Tween buffer. After elution with BBS/Tween buffer to minimize nonspecific protein absorption to the column, the bound antibody was eluted with 4 M guanidine HCl. This antibody preparation was purified by the same method.

Coagulation Assays—Prothrombin activity was assayed by measuring the acceleration of the clotting of prothrombin-deficient plasma (Factors II and VII deficient plasma, Sigma) by prothrombin (11). Abnormal prothrombin was measured using a modification of the E. carus and V. venom assay (25). The activation of prothrombin to thrombin was monitored by the generation of thrombin coagulant activity. A 100 μl aliquot of abnormal prothrombin was added to a solution containing 100 μl of 40 mM Tris-HCl (pH 8.1), 0.15 M NaCl and 100 μl of E. carus venom (1 mg/ml; Sigma) and incubated at 37°C for 5 min. Prothrombin-deficient plasma (Factor II- and VII-deficient plasma; 100 μl) was added to the resulting mixture and the clotting time was determined. Thrombin activity was measured by plotting the logarithm of the clotting time versus the logarithm of the thrombin concentration of standard solutions.

Quantitative Immuno-electrophoresis—Prothrombin and abnormal prothrombin were quantitated immunochemically using the Laurell rocket technique (25). Anti-prothrombin 1 antibodies (600 μg) were added to 10 ml of a 1% agarose solution at 95°C in barbital buffer. This solution was immediately poured onto a glass plate (13 × 8.2 cm) previously coated with a thin layer of 1% agarose. Immuno-
trophoresis was performed using an LKB Multiphor Model 2117 and an LKB power supply model 2103. The cooling plate was maintained at 0°C using a Neslab model RTE-8 refrigerated recirculating bath. Barbital buffer was used in the electrode troughs. Samples of 10 µl, applied to wells in the gel near the cathode, were run for 18 h at 2.5 V/cm constant voltage. The gel was washed with normal saline and distilled water, and stained with Coomassie Brilliant Blue R-250. The height of the precipitated line was measured by extrapolation to zero time hydrolysis. For quantitation of protein γ-carboxyglutamic acid, the protein was hydrolyzed in 2 M KOH for 2 h at 110°C (26).

Precipitin Analyses—Ouchterlony double immunodiffusion was performed using 1% agarose in barbital buffer. Patterns were examined after 24 h at 4°C. Quantitative precipitin analyses were performed in Microfuge tubes (Beckman). Tubes contained whole antisera (100 µl), prothrombin or abnormal prothrombin, as indicated, 1 mM CaCl₂ or 1 mM EDTA, as indicated, in a total volume of 250 µl. The tubes were mixed, held at 23°C for 1 h, and incubated at 4°C for 72 h. The precipitate was centrifuged and washed twice with 200 µl of 40 mM Tris·HCl (pH 8.1), 0.15 M NaCl at 4°C. The washed precipitate was dissolved in 10 µl of 1 M NaOH and the absorbance at 280 nm was determined. Antibody concentration was estimated using an E₂₈₀ of 14.0 for rabbit immunoglobulin.

Binding Studies—The binding of antianormal prothrombin antiserum or abnormal prothrombin-specific antibodies to [¹²⁵I]-labeled abnormal prothrombin and [¹⁴C]-labeled prothrombin was examined in the presence and absence of Ca(II) using the double antibody precipitation method to separate free and bound antigen. A 255-µl solution included 2.3 M NaCl, and the concentrations of the protein and the abnormal prothrombin antigen. This fraction was pooled, dialyzed exhaustively, and lyophilized prior to further purification. Because of difficulty in the accurate estimation of antigen concentration, the effect of 4 M guanidine HCl on abnormal prothrombin was inferred from experiments using prothrombin. When purified prothrombin was applied to the Sepharose-antiprothrombin 1 antibody column in the presence of buffers chosen to minimize nonspecific protein interaction with the column, the first peak eluted did not contain abnormal prothrombin antigen. The bound peak, eluted with 4 M guanidine HCl, contained approximately 4 mg of protein and the abnormal prothrombin antigen. This fraction was pooled, dialyzed exhaustively, and lyophilized prior to further purification. Because of difficulty in the accurate estimation of antigen concentration, the effect of 4 M guanidine HCl on abnormal prothrombin was inferred from experiments using prothrombin. When purified prothrombin was applied to the Sepharose-antiprothrombin 1 antibody column, eluted with 4 M guanidine HCl, and dialyzed against 40 mM Tris·HCl, 0.15 M NaCl, pH 8.0, all prothrombin coagulant activity could be recovered quantitatively. Thus, it would appear that guanidine HCl can be used successfully as an eluting agent without altering the coagulant or antigenic properties of the protein. In this step alone, a 50-fold purification of abnormal prothrombin was realized. To remove a high molecular weight contaminant from the abnormal prothrombin preparation, abnormal prothrombin was further purified by gel filtration on Sephacyl S200. The chromatogram was characterized by a large peak in the void volume, a small second peak, and a large symmetrical third peak; the latter was identified as abnormal prothrombin by quantitative immunoelectrophoresis. Overall, this preparative scheme offers a 550-fold purification of abnormal prothrombin antigen from plasma in 30% yield. The entire procedure can be completed in 4 days. A summary of the progress of purification is given in Table I.

Characterization of Abnormal Prothrombin—Purified abnormal prothrombin yielded a single band when examined by dodecyl sulfate-gel electrophoresis (Fig. 1). This material ap-
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Peared greater than 99% pure. Clotting activity of abnormal prothrombin as measured by the prothrombin assay was less than 3% of that expected from a similar quantity of purified bovine prothrombin, but had 86% of the activity of prothrombin when assayed by the E. carinatus assay. The small amount of activity observed in the prothrombin assay may be due to endogenous activity associated with abnormal protein (4).

The amino acid composition of bovine abnormal prothrombin is shown in Table II. The amino acid composition is comparable to those reported previously for abnormal prothrombin purified by other methods (4, 8). Of considerable interest to the current discussion is the observation that this preparation of abnormal prothrombin lacks γ-carboxyglutamic acid, as measured by alkaline hydrolysis and amino acid analysis. An upper limit of 0.5 mol of γ-carboxyglutamic acid/mol of abnormal prothrombin may be estimated, based upon the sensitivity of the amino acid analyzer in the detection of this amino acid. In comparison, bovine prothrombin contained 10 mol of γ-carboxyglutamic acid/mol of protein, as expected from the sequence data (27).

**TABLE I**

<table>
<thead>
<tr>
<th>Abnormal prothrombin purification</th>
<th>Protein (μg)</th>
<th>Total antigen (units)</th>
<th>Yielda</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bovine plasma</td>
<td>13,970</td>
<td>18,000</td>
<td>142.9</td>
<td>1.29</td>
<td>1.0</td>
</tr>
<tr>
<td>2. Barium citrate-absorbed plasma</td>
<td>10,440</td>
<td>12,606</td>
<td>100</td>
<td>1.20</td>
<td>1.0</td>
</tr>
<tr>
<td>3. DEAE-Sephadex</td>
<td>1,380</td>
<td>7,800</td>
<td>61.9</td>
<td>5.60</td>
<td>4.7</td>
</tr>
<tr>
<td>4. Affinity chromatography</td>
<td>21.0</td>
<td>5,520</td>
<td>43.8</td>
<td>263</td>
<td>219</td>
</tr>
<tr>
<td>5. Sephacryl S200</td>
<td>5.4</td>
<td>3,600</td>
<td>28.6</td>
<td>662</td>
<td>552</td>
</tr>
</tbody>
</table>

a Based upon barium citrate-absorbed plasma.

**TABLE II**

<table>
<thead>
<tr>
<th>Amino acid analysis of abnormal prothrombin</th>
<th>This worka</th>
<th>Ref. 10b</th>
<th>Ref. 4b</th>
</tr>
</thead>
<tbody>
<tr>
<td>residues/mole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>57.3</td>
<td>56.7</td>
<td>49.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>30.4</td>
<td>28.4</td>
<td>30.7</td>
</tr>
<tr>
<td>Serine</td>
<td>42.6</td>
<td>37.3</td>
<td>38.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>73.0</td>
<td>70.1</td>
<td>69.5</td>
</tr>
<tr>
<td>Proline</td>
<td>36.2</td>
<td>34.6</td>
<td>33.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>51.8</td>
<td>44.6</td>
<td>38.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>35.7</td>
<td>34.1</td>
<td>30.7</td>
</tr>
<tr>
<td>Cyclolane</td>
<td>N.D.</td>
<td>18.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Valine</td>
<td>30.0</td>
<td>34.5</td>
<td>36.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.4</td>
<td>7.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>19.7</td>
<td>19.4</td>
<td>17.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>48.7</td>
<td>42.7</td>
<td>43.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>19.1</td>
<td>16.0</td>
<td>20.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>18.9</td>
<td>18.7</td>
<td>24.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>9.6</td>
<td>10.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>30.8</td>
<td>33.0</td>
<td>26.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>38.5</td>
<td>38.2</td>
<td>29.7</td>
</tr>
<tr>
<td>γ-Carboxyglutamic acid</td>
<td>&lt;0.5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a Based upon a molecular weight of 74,000 and assuming carbohydrate content of 10.1%.
b Based upon a molecular weight of 72,000 and assuming carbohydrate content of 12.5%.
c N.D., not determined.
d Alkaline hydrolysis.

**FIG. 1. Abnormal prothrombin.** Purified abnormal prothrombin (30 μg) was evaluated by dodecyl sulfate-gel electrophoresis and stained with Coomassie Blue.

**FIG. 2. Evaluation of antiabnormal prothrombin antisera by Ouchterlony immunodiffusion.** A: center, antiabnormal prothrombin antisera; 1, abnormal prothrombin; 2, prothrombin; 3, prothrombin fragment 1; 4, Factor X; 5, Factor IX; 6, prothrombin fragment (12–44). B: center, antiprothrombin antisera. Peripheral wells were as described under A.
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Quantitative precipitation of abnormal prothrombin and prothrombin by antiabnormal prothrombin antisera. A, abnormal prothrombin (●, ○) in the presence of 1 mM CaCl₂ (●) or 1 mM EDTA (○). B, prothrombin (▲, △), in the presence of 1 mM CaCl₂ (▲) or 1 mM EDTA (△). C, abnormal prothrombin (●) and prothrombin (▲).

For comparison, antisera produced in rabbits injected with bovine prothrombin were studied (Fig. 2B). Antiprothrombin antisera also formed precipitating complexes with prothrombin and abnormal prothrombin, as previously reported (3). However, the existence of a spur between prothrombin and abnormal prothrombin indicates that antigenic determinants present in prothrombin do not exist in abnormal prothrombin.

The interaction of antiabnormal prothrombin antisera with abnormal prothrombin and prothrombin was investigated by quantitative precipitin analysis. As shown in Fig. 3, A and B, the presence or absence of 1 mM CaCl₂ did not alter the quantity of immunoprecipitate formed when abnormal prothrombin or prothrombin were employed as antigen. This is in contrast to parallel studies employing antiprothrombin antisera (10, 11) in which Ca(II) enhanced binding of prothrombin to antiprothrombin antisera. In addition, the quantitative precipitation of abnormal prothrombin by antiabnormal prothrombin antisera was greater than that observed using prothrombin (Fig. 3C). These results would suggest that a subpopulation of antiabnormal prothrombin antibodies is specific for abnormal prothrombin and does not bind to prothrombin.

Radioimmunoassay of Antiabnormal Prothrombin Antibodies—Abnormal Prothrombin Interaction—A radioimmunoassay was developed to measure the interaction of antiabnormal prothrombin antibodies in whole antisera with various antigens. 125I-Labeled abnormal prothrombin was prepared using Chloramine-T. This protein preparation migrated as a single protein peak on dodecyl sulfate-electrophoresis (Fig. 4), with greater than 95% of the radioactivity associated with the abnormal prothrombin peak.

In an experiment equivalent to that described in the legend to Fig. 3, the interaction of antiabnormal prothrombin antisera with 125I-labeled abnormal prothrombin and 125I-labeled prothrombin was studied in the presence and absence of 1 mM CaCl₂ using the radioimmunoassay (Fig. 5). The presence of CaCl₂ did not affect antibody-antigen interaction.

The binding of antiabnormal prothrombin antibodies in sera to a variety of protein antigens was further evaluated using the radioimmunoassay. In these experiments, the displacement of labeled abnormal prothrombin from the antiabnormal prothrombin antibodies by unlabeled antigen was studied (Fig. 6). Fifty per cent inhibition of 125I-labeled abnormal prothrombin-antibody binding was observed at molar concentrations of prothrombin, Factor IX, Factor X, and prothrombin fragment 1 that were 1.3-fold, 100-fold, 600-fold, and 600-fold, respectively, higher than that of abnormal prothrombin. No binding to prothrombin fragment (12-44) was observed. These results emphasize the immunochemical differences among the vitamin K-dependent proteins and, more importantly, indicate that the cross-reactivity of antibodies to abnormal prothrombin and prothrombin does not involve antigenic determinants on the fragment 1 portion of prothrombin.

Purification of Antibodies Specific for Abnormal Prothrombin—Antibodies specific for abnormal prothrombin were purified by sequential immunoabsorption using affinity chromatography. Because of limited quantities of abnormal prothrombin available, des-γ-carboxy-fragment 1 was prepared by the decarboxylation of fragment 1. Antiabnormal prothrombin antiserum (5 ml) was applied to a Sepharose-des-γ-carboxy fragment 1 column. The protein which failed to
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FIG. 5. Binding of abnormal prothrombin and prothrombin to antiabnormal prothrombin antisera. A, abnormal prothrombin (2 x 10^{-6} M) in the presence of 1 mM CaCl2 (△) or 1 mM EDTA (○). B, Prothrombin (2.2 x 10^{-7} M) in the presence of 1 mM CaCl2 (●) or 1 mM EDTA (○).

bind was discarded. The bound antibodies (2 to 3 mg) were eluted with 4 M guanidine HCl. After dialysis, these antibodies were applied to a Sepharose-prothrombin column. Antibodies which failed to bind to this second column (0.6 mg) were considered antiabnormal prothrombin-specific antibodies.

The interaction of antiabnormal prothrombin-specific antibodies with abnormal prothrombin and prothrombin was evaluated by radioimmunoassay. Using a direct binding assay, this antibody bound tightly to 125I-labeled abnormal prothrombin. This binding was not altered by the presence or absence of 1 mM CaCl2 or EDTA. In contrast, no binding to 125I-labeled prothrombin was detected (Fig. 7A) in the presence or absence of 1 mM EDTA. Similar results were observed using a competition assay. The binding of 125I-labeled abnormal prothrombin to antibody could be displaced by unlabeled abnormal prothrombin (Fig. 7B). However, concentrations of prothrombin in excess of 1600-fold of that of abnormal prothrombin demonstrated no displacement. In addition, prothrombin fragment 1 in 1500-fold molar excess of the abnormal prothrombin fragment (12-44).

FIG. 7. Interaction of antiabnormal prothrombin-specific antibodies with abnormal prothrombin and prothrombin. A, radioimmunoassay included 125I-labeled abnormal prothrombin in the presence of 1 mM CaCl2 or 1 mM EDTA (○) or 125I-labeled prothrombin in the presence of 1 mM CaCl2 or 1 mM EDTA (△) and antiabnormal prothrombin-specific antibody at the indicated concentration. Antibody-antigen complex formation is quantitated by the percentage of radiolabeled antigen bound to antibody. B, the interaction of antibody with abnormal prothrombin and prothrombin was studied using a competition assay. Antiabnormal prothrombin-specific antibody (3.5 x 10^{-8} M), 125I-labeled abnormal prothrombin (2 x 10^{-7} M), and unlabeled abnormal prothrombin (○) or prothrombin (●), as indicated, were incubated. Antibody-antigen complex formation was quantitated as the concentration of bound radiolabeled antigen divided by the concentration of free radiolabeled antigen. At 1600-fold excess of prothrombin to 125I-labeled abnormal prothrombin, interaction of antibody with prothrombin was not observed.
prothrombin concentration which effected 50% displacement of labeled antigen from antibody did not displace abnormal prothrombin from antibody (not shown).

The interaction of antiabnormal prothrombin-specific antibodies with plasma components was measured (Fig. 8) using a competition radioimmunoassay. Plasma from a calf treated with warfarin displaced labeled antigen from the antibody. In contrast, normal bovine plasma, normal human plasma, and human plasma from a patient treated with warfarin did not inhibit antibody-125I-labeled abnormal prothrombin interaction. From these experiments, it would appear that there is no significant cross-reactivity between these antibodies and abnormal prothrombin from human plasma. In addition, abnormal prothrombin is not a significant component of normal bovine plasma. An upper limit of the concentration of abnormal prothrombin in normal bovine plasma is about 1 ng/ml.

**DISCUSSION**

Abnormal prothrombin is a form of prothrombin that is not activated at a significant rate by activated Factor X and does not bind Ca(II). This form appears as a plasma protein in human and bovine blood in the face of vitamin K deficiency or the presence of vitamin K antagonists. Although the structure of this protein has not been studied in detail, abnormal prothrombin is known to lack γ-carboxyglutamic acid (5, 6). It has otherwise been assumed that, with the exception that abnormal prothrombin contains glutamic acid residues instead of γ-carboxyglutamic acid residues, the primary structure, extent of glycosylation, and molecular size of abnormal prothrombin and prothrombin are identical. In the current study, we have developed a rapid, facile method for the purification of bovine abnormal prothrombin. Using this material as immunogen, antibodies were raised in rabbits and an antibody isolated.

As a central feature of the purification scheme for abnormal prothrombin employed is the ability of antiprothrombin antibodies to bind to both prothrombin and abnormal prothrombin. Since abnormal prothrombin does not bind to insoluble barium salts, a major strategy of purification of the vitamin K-dependent plasma proteins cannot be exploited. Thus, we have employed affinity chromatography using antiprothrombin 1 antibodies coupled to agarose beads to bind selectively to the abnormal prothrombin. Although several elution systems were tried, guanidine HCl allowed quantitative recovery of protein, abnormal prothrombin antigen, and potential thrombin activity. Parallel experiments with bovine prothrombin confirmed that no detectable alterations in prothrombin structure or activity were caused by the brief exposure to 4 M guanidine HCl.

Purified bovine abnormal prothrombin was obtained in high yield and of quality suitable for use as immunogen. Analysis of this protein preparation indicated that abnormal prothrombin contained no detectable γ-carboxyglutamic acid. Although other authors have reported partially carboxylated forms of prothrombin in plasma obtained from humans (28, 29) or steers (29, 30) treated with vitamin K antagonists, the preparative procedure described yielded a product which appears homogeneous and without γ-carboxyglutamic acid.

Antibodies raised to abnormal prothrombin offer an immunologic approach to the comparison of the structure of abnormal prothrombin and prothrombin. These studies parallel our immunochemical evaluation of prothrombin (11, 12, 31). Recently, we have purified an antibody subpopulation directed against the γ-carboxyglutamic acid-rich region of prothrombin from anti-prothrombin antisera (11). Some of these antibodies are conformation-specific for antigenic determinants whose structure is stabilized by metal ions, including Ca(II) (12). In a separate study, antibodies which bind to the prothrombin-Ca(II) binary complex have been purified (31).

It now appears that these antibodies are directed against determinants in the fragment 1 region of prothrombin and that the NH₂-terminal third of prothrombin undergoes a structural transition that is induced by Ca(II). Thus, it is of interest to observe that the antiabnormal prothrombin antisera and the antibody subpopulation specific for abnormal prothrombin bind very poorly to prothrombin fragment I (M₄ = 23,000). Although fragment 1 contains 10 γ-carboxyglutamic acid residues near the NH₂ terminus, it might be expected that the fragment 1 regions of the prothrombin and abnormal prothrombin, representing 156 amino acid residues which are otherwise identical, might share antigenic determinants unless the major antigenic determinants include γ-carboxyglutamic acid. However, antibodies directed against region 12-44 of prothrombin bind poorly to fragment 12-44 and not at all to γ-carboxyglutamic acid. The absence of common antigenic determinants, in the presence or absence of Ca(II), suggests that either γ-carboxyglutamic acid residues confer significant conformational stability to a unique three-dimensional structure of prothrombin or that much of the fragment 1 region is not exposed. The latter appears unlikely since two-thirds of the antiprothrombin antibodies were directed against the fragment 1 region, representing one-third of the protein. We can thus conclude that there are generalized differences in the tertiary structure of the fragment 1 regions of prothrombin and abnormal prothrombin and that the removal of metal ions with EDTA does not make prothrombin structure approach that of abnormal prothrombin.

The antiabnormal prothrombin antibodies bound specifically to abnormal prothrombin. At concentrations of prothrombin that were in 1000-fold excess of abnormal prothrombin, no detectable antibody-prothrombin interactions were observed. Thus, this antibody reagent is highly specific for the decarboxylated form of prothrombin. Preliminary measurements of the levels of decarboxylated forms of prothrombin in normal bovine plasmas have suggested that these forms are not circulating plasma proteins under normal conditions. Rather, they appear with alterations of vitamin K metabolism.
which inhibit the vitamin K-dependent hepatic carboxylase. At present, we do not know whether this antibody, which binds specifically to abnormal prothrombin, or anti (12-44), which binds specifically to prothrombin (11), can be used to distinguish various partially carboxylated prothrombin species which occur naturally. These species offer an approach to the study of the structure-function relationship of various $\gamma$-carboxyglutamic acid residues. The antibody reagents described may be useful in purifying and characterizing these forms of prothrombin. In addition, preliminary experiments have indicated that antiabnormal prothrombin-specific antibodies bind to some proteins in the crude homogenate of normal liver and isolated microsomal extracts from normal liver. These cross-reactive proteins are likely to be precursors involved in the synthesis of prothrombin. As such, the antiabnormal prothrombin-specific antibodies should prove useful in the evaluation and purification of proteins involved in prothrombin biosynthesis.

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