A Functional Assay of Protein C in Human Plasma

By Núria Sala, Whyte G. Owen, and Désiré Collen

A three-step spectrophotometric assay was developed for measuring functional protein C (PC) in human plasma. The assay is based on: (1) adsorption of citrated platelet-poor plasma on barium citrate and elution of the vitamin K-dependent factors with EDTA; (2) activation of PC by incubation of the mixture of vitamin K-dependent factors with a complex of thrombin and its endothelial cell cofactor, thrombomodulin; (3) addition of antithrombin III and heparin to the system to inhibit thrombin and other coagulation enzymes generated during incubation and measurement of the activated PC with a synthetic (chromogenic) substrate. The assay appears to be specific for PC because: (a) PC-depleted plasma (by immunoadsorption) is inactive; (b) addition of purified PC to PC-depleted plasma reconstitutes its activity; and (c) no enzymatic activity is generated in the absence of the thrombin–thrombomodulin complex.

PROTEIN C (PC) is a vitamin K-dependent zymogen of the serine protease-activated protein C (PCa).1 Activation of PC by thrombin is markedly enhanced by the endothelial cell surface protein thrombomodulin.2 Activated PC exerts an anticoagulant activity3 by inhibiting factors Va and VIIIa4,5 and stimulates release of plasminogen activator in dogs in vivo.6 Measurement of PC in human plasma by immunoassay7,8 has revealed several kindred having congenital PC deficiency associated with recurrent thrombosis and several clinical conditions associated with acquired PC deficiency.

Functional assays of PC in plasma have been hampere-red by the low concentration of the zymogen, the endogenous inhibitors of PCa, and the lack of specific activation methods. In the present study, we have approached these problems by extracting PC with barium citrate, activating the partially purified PC with thrombin–thrombomodulin complex, and then measuring the PCa with a synthetic chromogenic substrate. The assay appears to be specific, reproducible, and capable of detecting decreased levels in patients with congenital or acquired PC deficiency.

MATERIALS AND METHODS

Materials

CNBr-activated Sepharose and protein A-Sepharose were from Pharmacia (Uppsala, Sweden); polyethylene glycol 6,000 from Merck (Schuchard, Germany); Triton X100 from Packard Instrument Company Inc. (Downers Grove, IL); bovine albumin from Povite, Poviet Produce BV (Oss, The Netherlands); and immunglobulin G (IgG) from the Red Cross (Leuven, Belgium). The chromogenic substrates H-D-phenylalanyl-pipecolyl-L-arginine-pNA (S-2366), H-D-isoleucyl-L-prollyl-L-arginine-pNA (S-2238), H-D-valyl-L-leucyl-L-arginine-pNA (S-2266), H-D-phenylalanyl-pipecolyl-L-arginine-pNA (S-2238), and L-pyro-glutamyl-L-pro-lyl-L-arginine-pNA (S-2366) were gifts from KabiVitrum (Amsterdam, The Netherlands, courtesy of Mr. Kortmann).

Bovine a-thrombin was purified from Topostase (Roche, Brussels, Belgium) as described previously.11 The purified enzyme had a specific activity of 2,400 US U/mg; it was stored at –80°C in aliquots of 400 US U/ml in 0.15 M NaCl, 0.1 M Na2HPO4 buffer, pH 6.5, containing 1 mg bovine albumin/ml.

Thrombomodulin was prepared by extraction of homogenized rabbit lungs with Triton X100 and chromatography of the crude extract on DIP-thrombin-Sepharose as described previously.2 Thrombomodulin concentration was determined by titration with thrombin.12 Antithrombin III and high-affinity heparin were purified as described elsewhere.13 Human PC was purified from human factor IX complex (Hyland Therapeutic Division, Glendale, CA; courtesy of Dr. R. A. De Vreker) by the method of Suzuki et al.14 Fractions containing PC were identified by electromunoassay.

The protein content was determined by the method of Lowry and by amino acid analysis. The final material was obtained as a solution containing 340 µg PC/ml, and only traces of factors VII, IX, and X and no detectable prothrombin. It showed a single broad band (mol wt 60,000) and, after reduction, two bands (mol wt 43,000 and 24,000) on SDS-gel electrophoresis.

Initial experiments were carried out with purified PC preparations, kindly given to us by Dr. J. Stenflo, Malmö, Sweden, and Dr. R. M. Bertina, Leiden, The Netherlands.

From the Center for Thrombosis and Vascular Research, Department of Medical Research, University of Leuven, Belgium.

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Address reprint requests to Dr. D. Collen, Center for Thrombosis and Vascular Research, Department of Medical Research, University of Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

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Rabbit antiserum to human PC was obtained by immunization with three biweekly multiple site subcutaneous injections of 50 μg of antigen dissolved in 0.5 ml of 0.15 M NaCl and emulsified with 0.5 ml of Freund's complete adjuvant. Serum collection was started 1 wk after the last injection. The IgG fractions of the antiserum were isolated by affinity chromatography on protein A-Sepharose and coupled to CNBr-activated Sepharose and used to prepare protein C-depleted plasma by immunoadsorption of pooled normal plasma. The immunoadsorbed plasma had normal levels of clotting factors, except fibrinogen, which was also removed.

Functional Assay of Protein C

All blood samples were collected in trisodium citrate anticoagulant (final concentration 0.012 M) and centrifuged for 10 min at 1,500 g to obtain platelet-poor plasma.

Barium citrate adsorption. The barium citrate adsorption and elution were adapted from the procedure of Aronson et al. In a plastic centrifuge tube, 80 μl of 1 M BaCl2 in 0.3 M Tris-HCl buffer, pH 8.0, was added to 1 ml of plasma precooled to 4°C. The mixture was stirred at 4°C for 10 min and then centrifuged for 15 min at 10,000 g and 4°C. The supernatant was discarded, and the precipitate was washed with 1 ml of 0.15 M BaCl2 in 0.02 M Tris-HCl, pH 8.0. The proteins were eluted from the barium citrate precipitate by dissolution in 0.5 ml of 0.1 M Na-EDTA (pH 6.0). To remove the EDTA, the dissolved proteins were precipitated by adding 50 μl of a 2% IgG solution (final concentration 1 mg/ml) and 0.5 ml of a 40% solution of polyethylene glycol 6,000 (final concentration 20%). After incubation for 15 min at room temperature, the precipitated proteins were isolated by centrifugation at 40,000 g for 15 min and dissolved in 0.5 ml of 2 mM trisodium citrate, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5. Aliquots of 125 or 250 μl were used immediately for the assay of PC or were kept at -20°C.

Protein C activation. To a 3-ml plastic test tube containing 250 μl of the barium citrate eluate were added: (1) 150 μl of a buffer solution containing 0.1 M NaCl, 0.17 M Tris-HCl, 0.33% Triton X100, 3.3 mg/ml bovine albumin, and 33 mM CaCl2, pH 8.0; and (2) 100 μl of 100 nM thrombin–thrombomodulin complex. This complex was prepared by mixing 1 volume of thrombin (200 nM, or 20 US U/ml) in 0.1 M NaCl, 0.05 M Tris-HCl, pH 8.0, with 1 volume of thrombomodulin (200 nM in the same buffer). The mixture was incubated at 37°C for 60 min to obtain maximal activation of protein C. Thus, the composition of the activation mixture was: thrombin 20 nM, thrombomodulin 20 nM, Ca2+ 10 mM, albumin 1 mg/ml, Triton X100 0.1% (v/v) in 0.125 M NaCl, 0.07 M Tris-HCl, pH 8.0.

Measurement of activated Protein C. After activation of PC was complete, 200 μg of antithrombin III, 0.4 U of high-affinity heparin in 100 μl Tris-NaCl buffer, pH 7.6, was added and the mixture was incubated at 37°C for 10 min. In a 1-cm cuvette, 200 μl of the PCa mixture was diluted with 750 μl of 0.1 M NaCl, 0.05 M Tris-HCl, 0.01 M CaCl2, pH 8.0. Substrate (50 μl of 4 mM S-2266) was then added, and the release of paranitroanilide was determined by recording A5 at approximately 2 min. Change in A5 was converted to PC concentration (percent of normal) by using a calibration curve constructed with mixtures of normal and PC-depleted plasmas. Blank hydrolysis rates, determined by omitting thrombin–thrombomodulin from the activation mixtures, were not significant.

Protein C was also measured in plasma and in purified preparations by a standard four-compartment radioimmunoassay.

RESULTS

Activation of Purified Protein C

Figure 1 shows the activation of different concentrations of PC with a mixture containing 20 nM thrombin–thrombomodulin complex and 10 mM CaCl2. The activation was complete within 1 hr, and the yield of PCa was proportional to PC concentration. In the presence of CaCl2, both thrombin and thrombomodulin were required for activation. In the absence of CaCl2, thrombin alone could activate PC, but only at much higher concentrations.

The activation rate of PC with increasing concentrations of thrombin–thrombomodulin complex is shown in Fig. 2A; the influence of CaCl2 concentration on the activation rate of PC is shown in Fig. 2B. Maximal activation is obtained after 1 hr of incubation at 37°C with 20 nM thrombin–thrombomodulin complex and 10 mM CaCl2, and these conditions were chosen for routine activations.

Catalytic Efficiencies of PCa and Thrombin for Synthetic Chromogenic Substrates

To obtain the optimal substrate for the measurement of PCa, the kinetic constants of PCa and thrombin toward some synthetic substrates (S-2238, S-2266, S-2288, S-2366) were determined from Lineweaver-Burk plots (Table 1). The results indicate that S-2266 is the most specific substrate for PCa (relative to thrombin); it was used for subsequent experiments.

Activation of Protein C in the Barium Citrate Eluate

The time course of the activation of PC obtained by adsorption of normal plasma with barium citrate was
Table 1. Kinetic Constants of PCa and Thrombin Toward S-2238, S-2288, S-2266, and S-2366

<table>
<thead>
<tr>
<th>S-2238</th>
<th>900</th>
<th>77</th>
<th>0.086</th>
<th>6.5</th>
<th>160</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-2288</td>
<td>440</td>
<td>77</td>
<td>0.18</td>
<td>4.1</td>
<td>130</td>
<td>32</td>
</tr>
<tr>
<td>S-2266</td>
<td>360</td>
<td>22</td>
<td>0.061</td>
<td>380</td>
<td>19</td>
<td>0.050</td>
</tr>
<tr>
<td>S-2366</td>
<td>800</td>
<td>160</td>
<td>0.20</td>
<td>170</td>
<td>290</td>
<td>1.70</td>
</tr>
</tbody>
</table>

not lose PC activity. When increasing amounts of purified PC were added to PC-depleted plasma, approximately 85% was recovered as PCa. The average recovery of prothrombin was 95%.

Reproducible results were only obtained when strict temperature control during the barium citrate adsorption was observed.

Reproducibility of the Measurement of Protein C in Plasma

The reproducibility of the assay was measured by performing, for 4–6 consecutive days, triplicate measurements of a normal plasma pool, a pool of plasma from dicoumarol-treated patients (prothrombin level 25% of normal), and a plasma reconstituted by mixing 1/4 PC-depleted plasma with 3/4 pooled normal plasma (Table 2).

Plasma Protein C Levels in Healthy Individuals, Dicoumarol-Treated Patients, and in Congenital PC Deficiency

The levels of functional PC in the plasma from 13 men and 10 women were 97% ± 15% (mean ± SD) of the value found for the normal plasma pool (Table 3). There was no significant difference between men (99% ± 14%) and women (94% ± 16%).

The functional PC levels in 14 patients on stabilized dicoumarol therapy (prothrombin level 25% ± 7%) was 40% ± 8% and their PC antigen level 2.0 ± 0.3 μg/ml (71% ± 11%). In 3 patients with previously established congenital PC deficiency, the functional PC level was 37% ± 9% and the PC antigen level 1.8 ± 0.3 μg/ml (64% ± 11%).

Correlation Between the Functional Assay and a Radioimmunoassay for Protein C

Figure 4 illustrates the correlation between PC determined by the functional assay and by a radioimmunoassay. A linear correlation was found with $r = 0.86$.

DISCUSSION

With the recent development of immunoassays for PC in human plasma, congenital and acquired defi-
ciencies of PC have been found. However, the lack of specific functional assays has thus far precluded the detection of abnormal PC molecules. In the present study, we have developed a functional assay of PC extracted from plasma by adsorption to barium citrate, activation of the extracted PC with thrombin–thrombomodulin complex, and then measurement of PCa with a chromogenic substrate. Measurement of PCa directly in plasma, either by clotting assays (activated partial thromboplastin time or factor V assay after activation of PC) or with synthetic substrates, did not yield reproducible results, probably due to the presence of PC inhibitors in plasma. Therefore, PC was extracted from plasma by adsorption on barium citrate. The recovery of PC in barium citrate eluates was 85% and sufficiently reproducible, provided the temperature was carefully controlled during the adsorption. PCa could be measured adequately with a synthetic chromogenic substrate. The extraction, activation, and measurement are relatively simple and can be carried out on several samples within a single working day. Although we have preferred to carry out most experiments with highly purified reagents, control experiments with commercial grade thrombin, antithrombin III, and heparin yielded virtually identical results (not shown).

The assay developed in the present study is specific for PC, and a linear correlation is observed between PCa activity and PC antigen. The assay is sufficiently sensitive to detect patients with congenital PC deficiencies (<50% of normal) or with lowered PC levels secondary to dicoumarol treatment. However, it should be stressed that measurements of PCa with a synthetic substrate do not necessarily reflect its biologic activity.

Table 2. Reproducibility of Protein C Assay in Plasma

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Antigen (µg/ml)</th>
<th>Functional Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool of normals</td>
<td>23</td>
<td>2.8 ± 0.6</td>
<td>97 ± 15</td>
</tr>
<tr>
<td>Pool of dicoumarol-treated patients</td>
<td>13</td>
<td>2.9 ± 0.7</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>Mixture of 3/4 PC-depleted and 1/4 normal plasma</td>
<td>10</td>
<td>2.8 ± 0.6</td>
<td>94 ± 16</td>
</tr>
</tbody>
</table>

Fig. 3. Amidolytic activity generated from mixtures of normal and PC-depleted plasma. Experimental conditions were as described in Materials and Methods. Vertical bars represent SEM of 6 measurements.

Fig. 4. Correlation of PC determined by the functional assay and by a radioimmunoassay. (□) Plasma from healthy individuals and hospitalized patients; (■) patients on stabilized dicoumarol therapy; and (△) mixtures of normal and PC-depleted plasma pools. The correlation line was constructed without considering the values of dicoumarol-treated patients.

Table 3. Protein C Levels in Plasma

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Antigen (µg/ml)</th>
<th>Functional Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>23</td>
<td>2.8 ± 0.6</td>
<td>97 ± 15</td>
</tr>
<tr>
<td>Men</td>
<td>13</td>
<td>2.9 ± 0.7</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>Women</td>
<td>10</td>
<td>2.8 ± 0.6</td>
<td>94 ± 16</td>
</tr>
<tr>
<td>Dicoumarol-treated patients</td>
<td>14</td>
<td>2.0 ± 0.3</td>
<td>40 ± 8</td>
</tr>
</tbody>
</table>

PCa activity and PC antigen. The assay is sufficiently sensitive to detect patients with congenital PC deficiencies (<50% of normal) or with lowered PC levels secondary to dicoumarol treatment. However, it should be stressed that measurements of PCa with a synthetic substrate do not necessarily reflect its biologic activity.
We anticipate that, with the availability of a functional assay for PC, individuals with PC molecules that cannot undergo normal activation or that lack catalytic activity will be detected. The assay may also be of use for the further investigation of the pathophysiologic role of PC in the regulation of hemostasis and thrombosis.

ACKNOWLEDGMENT

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