Mechanism of Action of Human Activated Protein C, a Thrombin-Dependent Anticoagulant Enzyme

By Richard A. Marlar, Alice J. Kleiss, and John H. Griffin

Human protein C was purified, and the mechanism of action of activated protein C as an anticoagulant in plasma was studied. Protein C was purified from commercial factor IX concentrate by DEAE-Sephadex and dextran sulfate-Sepharose chromatography and preparative polyacrylamide gel electrophoresis. Purified protein C appeared homogeneous at 62,000 mol wt on nonreduced SDS-polyacrylamide gels and, following reduction, protein C gave two polypeptide chains of 40,000 and 22,000 mol wt. Protein C was activated by immobilized trypsin or thrombin or by soluble thrombin. Activated protein C markedly prolonged the prothrombin time and the activated partial thromboplastin time of normal plasma, but had no effect on the thrombin time. Activated protein C exhibited amidolytic enzyme activity. DFP inhibited both the amidolytic and anticoagulant activities. Activated protein C was added to human plasma in the presence or absence of phospholipid or calcium. This mixture was incubated for 3 min at 37°C, diluted with EDTA, and the remaining clotting activity of each of the coagulation factors was determined using appropriate deficient plasmas. Activated protein C inactivated >80% of factor V and factor VIII under these conditions, whereas factors I, II, X, VII, IX, XI, and XII, prekallikrein, and high molecular weight kininogen were not affected. Both phospholipid and calcium were required for potent inactivation of factors V and VIII in plasma. When partially purified factor VIII was activated by thrombin, it was 30 times more susceptible to inactivation than nonactivated factor VIII. These results suggest that by inactivating both factor V and factor VIII, the enzymatically active form of protein C may serve as a major physiologic regulator of cofactors of coagulation during hemostasis and thrombosis.

HUMAN PROTEIN C is a vitamin-K-dependent serine protease zymogen and is homologous with other known vitamin-K-dependent serine proteases.1 Activated protein C is anticoagulant, since it prolongs the clotting time of plasma in various clotting assays.2 Bovine protein C has been well characterized functionally and physiochemically,3,4 while less is known about the human molecule. Kisel1 reported the isolation and partial characterization of human protein C. He showed that it could be activated by limited proteolysis by thrombin and that this activated form exhibited anticoagulant activity. Human protein C appears to be structurally and functionally similar to bovine protein C, although the anticoagulant activity of each of these proteins is highly species specific.

Activated bovine protein C, then designated autoprothrombin II-A, was described 21 yr ago by Seegers and coworkers.3 A decade later, Marciniak1 reported several observations on human autoprothrombin II-A and noted it was species specific. In 1976, Stenflo7 isolated a protein, referred to as protein C, which Seegers et al.8 showed was immunologically indistinguishable from autoprothrombin II-A. The anticoagulant activity of bovine activated protein C was initially ascribed to a competitive inhibition of factor Xa.9,10 Various investigators have shown that bovine activated protein C enzymatically inactivates bovine factors V and VIII.3,10,11 Walker12 recently reported that protein S potentiates the anticoagulant activity of bovine activated protein C.

In this article, the mechanism of anticoagulant activity of human activated protein C in plasma and the conditions necessary for expression of this activity are described.*

MATERIALS AND METHODS

Materials

All chemicals from commercial sources were the best grade available. DEAE-Sephadex A50, CNBr-activated Sepharose 4B, and Sepharose 4B were products of Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Dextran sulfate-Sepharose was made according to Kissel.1 Purified α-thrombin, prepared from purified human prothrombin using Echis carinatus venom,11 was coupled to CNBr-activated Sepharose 4B following the manufacturer’s instructions. The chromogenic substrates, S-2238 (D-Phe-Pip-Arg-paranitroanilide) and S-2160 (Bz-Phe-Val-Arg-paranitroanilide) were purchased from Kabi Ab, Stockholm, Sweden. The various deficient plasmas were purchased from George King Biomedical, Overland, Kans. Commercial rabbit brain thromboplastin was obtained from Ortho Diagnostics, Raritan, N.J., and rabbit brain cephalin was purchased.


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Anticoagulant Activity of Activated Protein C

Normal human plasma (500 μl) was incubated with activated protein C (0.5 μg/ml final concentration) plus cephalin (58 μg/ml) and 4 mM calcium chloride at 37°C in a total volume of 550 μl. The reaction was initiated by adding 50 μl of 5 mM EDTA or by the addition of purified goat anti-protein-C gamma globulin fraction from a goat antiserum. This mixture was then assayed for the presence of the various clotting factor activities using appropriate deficient human plasmas. The clotting activity of factors II, V, VII, and X were assayed using a prothrombin time assay, and factors VIII, IX, XI, XII and prekallikrein and high molecular weight kininogen were assayed by a kaolin-activated partial thromboplastin time. In some experiments, buffer replaced protein C or cephalin or calcium in the reaction mixture.

Factor VIII Activation and Inactivation

Commercial human factor VIII concentrate (Hemophil), further purified by gel filtration, was used as a source of factor VIII. Lyophilized factor VIII concentrate (500 clotting units) was reconstituted to 5 ml with 0.01 M imidazole-HCl, 0.15 M sodium chloride, 0.02% sodium azide, pH 7.4, and applied at 37°C to a Sepharose-4B column (2.5 x 65 cm) equilibrated in 0.01 M imidazole-HCl, 0.15 M sodium chloride, 0.02% sodium azide, pH 7.4. Factor VIII clotting activity eluted in the void volume and was free of fibrinogen. This partially purified factor VIII (8 clotting units/ml final) was activated in a total volume of 1 ml using α-thrombin-Sepharose beads (200 μl) that were then removed from the mixture by centrifugation. The progress of factor VIII activation was monitored by an APTT assay using factor-VIII-deficient plasma. Activated protein C (1.8 μg/ml), cephalin (58 μg/ml), and calcium (4 mM final concentration) were added to either the nonactivated or thrombin-activated factor VIII in a final volume of 550 μl, and the remaining factor VIII clotting activity was determined using the APTT assay.

RESULTS

Purification of Human Protein C

Human factor IX concentrate was the source of human protein C. The DEAE-Sephadex chromatography separated the majority of protein C from the other vitamin-K-dependent coagulation proteins, as well as removed the bulk of other proteins. Dextran sulfate-Sepharose chromatography removed residual factors IX and VII, as well as the majority of the prothrombin. Following the preparative polyacrylamide gel electrophoresis procedure, the purified protein C contained no measurable (<0.1%) prothrombin, factor VII, factor IX, or factor X, as measured by clotting assays. On 10% sodium dodecyl sulfate polyacrylamide gels in the absence of reducing agent, protein C appeared as a single band with an apparent 62,000 mol wt, however, in the presence of a reducing agent, protein C formed two bands with mobilities representing 40,000 and 22,000 mol wt on SDS-polyacrylamide gels. Thus, human protein C, like the bovine molecule, consists of two polypeptide chains linked by disulfide bonds, as previously reported by others.

Activation of Protein C

Human protein C was activated using soluble α-thrombin, α-thrombin-Sepharose beads, and commercial trypsin-agarose beads. Thrombin was generated by partially purified human prothrombin using Echis carinatus venom and isolated as previously described. The purified thrombin was coupled to CNBr-activated Sepharose according to manufacturer’s instructions.

To monitor the activation of protein C, the amidolytic activity was determined using the chromogenic substrate, S-2238. In the assay, 60 μl of the sample was added to 600 μl 0.3 mM amidolytic substrate in buffer 0.05 M Tris, 0.1 M NaCl, pH 8.3. The rate of absorbance change at 405 nm/min was determined.

Inactivation of Activated Protein C

Activated human protein C (50 μg/ml) was incubated for 10 min with 5 mM diisopropylfluorophosphate in a total volume of 2 ml. The mixture was then dialyzed to remove excess DFP.
Activation of Protein C

Human protein C was activated with thrombin-Sepharose beads. Protein C amidolytic activity increased with time and reached a maximum within 60 min (Fig. 1). After removing the thrombin-Sepharose by centrifugation, activated protein C remained stable, and no thrombin activity was detected in clotting assays or in assays of S-2160 amidolytic activity. The latter assay was particularly useful since S-2160 is an excellent substrate for thrombin, but a very poor one for human activated protein C. For example, S-2238 was hydrolyzed at least 50 times faster than S-2160 by human activated protein C when each oligopeptide substrate was tested at 0.3 mM. Activated protein C generated in this manner functioned as an anticoagulant in normal plasma. When activated protein C (3 μg/ml final concentration) was added to an APTT assay in normal plasma, the clotting time was prolonged from 47 sec to 249 sec.

Anticoagulant Activity of Protein C

The effect of activated protein C (3 μg/ml) in the presence of phospholipid and calcium on some clotting assays is shown in Table 1. Activated protein C significantly prolonged the prothrombin time, the activated partial thromboplastin time, and the partial thromboplastin time (Table 1). Activated protein C did not affect the thrombin time using normal plasma and human thrombin (Table 1).

The effect of activated protein C (3 μg/ml) on the

<table>
<thead>
<tr>
<th>Clotting Time (sec)</th>
<th>Activated Protein C</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>Prothrombin time</td>
<td>68</td>
<td>24</td>
</tr>
<tr>
<td>Activated partial thromboplastin time</td>
<td>616</td>
<td>57</td>
</tr>
<tr>
<td>Partial thromboplastin time</td>
<td>&gt;1200</td>
<td>247</td>
</tr>
<tr>
<td>Thrombin time</td>
<td>17.9</td>
<td>18.2</td>
</tr>
</tbody>
</table>

Activated protein C (3 μg/ml final), cephalin, and calcium preincubated for 30 sec prior to initiating the assay.

Table 1. Effect of Activated Protein C on the Common Coagulation Assays

![Fig. 1. Activation of protein C by insolubilized α-thrombin. Amidolytic activity of activated protein C was measured spectrophotometrically with the chromogenic substrate, S-2238. At various times, aliquots were withdrawn from the reaction mixture, centrifuged to remove the thrombin-Sepharose, diluted, and assayed for amidolytic activity (solid circles) or unactivated protein C (open circles). See Materials and Methods section for details.](http://www.bloodjournal.org)

![Fig. 2. Effect of activated protein C on coagulation factors in normal plasma. Activated protein C (3.0 μg/ml final) was combined with citrated normal human plasma (500 μl) in the presence of cephalin (58 μg/ml final) and calcium ions (4 mM final) in a total volume of 600 μl. After 3 min at 37°C, EDTA or purified anti-protein C antibodies was added, and dilutions of the mixture were assayed for individual coagulation factors. The height of each bar represents percent clotting activity compared to the control (open bars). Plasma plus activated protein C is represented by bars with diagonal lines.](http://www.bloodjournal.org)
factors in plasma. Only protein C that had been activated destroyed the clotting activities of factors V and VIII in plasma.

Both phospholipid and calcium ions were needed as cofactors for activated protein C to exhibit potent anticoagulant activity in various coagulation assays. Therefore, these two cofactors were tested to determine their influence on factor V and factor VIII inactivation (Table 2). For optimum inactivation of each clotting factor, both phospholipid and calcium were required in conjunction with activated protein C, since no significant loss of factors V or VIII activity was observed in the absence of either cofactor.

**Factor VIII Activation and Inactivation**

Factor VIII was activated by insolubilized thrombin to increase greatly its procoagulant activity. Partially purified factor VIII was prepared by gel filtration and was free of clottable fibrinogen. In 15 min with thrombin-Sepharose beads, the clotting activity of factor VIII increased 20-fold from 8 to 170 U/ml (Fig. 4). Following removal of the thrombin-Sepharose and upon the addition of activated protein C, phospholipid, and calcium at 15 min (arrow, Fig. 4), the clotting activity of thrombin-activated factor VIII (factor VIIIa) rapidly disappeared. Calcium and phospholipid were required for this rapid inactivation of factor VIIIa. When the data for inactivation of factor VIII and factor VIIIa were plotted on a semi-log graph of log residual activity versus time, linear plots resulted

![Graph of clotting activity](image-url)

**Table 2. Influence of Protein C, Activated Protein C, DIP-Protein C, and the Cofactors, Cephalin and Calcium, on the Clotting Activity of Factor V and Factor VIII in Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Factor V</th>
<th>Factor VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 (0.67)</td>
<td>100 (1.4)</td>
</tr>
<tr>
<td>Protein C</td>
<td>100 (0.67)</td>
<td>107 (1.5)</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>16 (0.11)</td>
<td>19 (0.27)</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>100 (0.67)</td>
<td>100 (1.4)</td>
</tr>
<tr>
<td>calcium absent</td>
<td>94 (0.63)</td>
<td>100 (1.4)</td>
</tr>
<tr>
<td>DIP-protein C</td>
<td>100 (0.67)</td>
<td>100 (1.4)</td>
</tr>
</tbody>
</table>

* Plasma was incubated with test sample (3 μg/ml final) for 3 min at 37°C and then assayed for the remaining clotting factor activity using deficient human plasma. 4 mM CaCl₂ and 58 μg/ml cephalin were present unless otherwise indicated. Buffer replaced protein C in the control.

† The values in parentheses are the observed activities in clotting units/ml.

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PROTEIN C

Fig. 5. Comparison of kinetics of inactivation of factor VIIIa and factor VIII by activated protein C. Nonactivated factor VIII (open circles) and thrombin-activated factor VIII (solid circles) were inactivated with activated protein C (1.8 µg/ml final concentration). At various time points, aliquots were removed and assayed for residual factor VIII activity. The data were plotted in a semi-log plot of log factor VIII activity remaining versus time. See Materials and Methods section for details and assay method.

(Fig. 5). By comparing the slopes of these plots, it was apparent that the factor VIIIa was 30 times more rapidly inactivated than the untreated factor VIII. The initial phase of factor VIII inactivation may be due to contamination of factor VIII with approximately 1% of factor VIIIa, which is rapidly destroyed by activated protein C.

DISCUSSION

Human protein C that was prepared in our lab from commercial factor IX concentrate appears indistinguishable from that isolated from plasma by Kisiel. Moreover, it appears that human protein C is very similar to the bovine protein. Both molecules contain two disulfide-linked polypeptide chains with similar molecular weights, amino acid and carbohydrate compositions, and sequence homology. Both are anticoagulants in their respective plasmas. However, the species specificity of protein C is such that the human activated protein C is not very anticoagulant in bovine plasma and the activated bovine molecule is not very anticoagulant in human plasma. The human enzyme is shown here to function in plasma by inactivating factors V and VIII.

Human activated protein C functions as a potent anticoagulant in human plasma by inactivating the coagulation cofactors V and VIII. The rate of inactivation of these cofactors in plasma is rapid, since greater than 80% of their activity is destroyed in 3 min by 3 µg/ml of activated protein C. Human protein C must be proteolytically activated to function as an anticoagulant. Activated protein C loses its anticoagulant properties upon incubation with the serine protease inhibitor, DFP, implying that protein C functions enzymatically by proteolytic inactivation. Both phospholipid and calcium ions are needed as cofactors for the expression of anticoagulant activity.

The potent inactivation of factors V and VIII in plasma by activated protein C (Figs. 2 and 3) is entirely sufficient to explain the prolongation of the partial thromboplastin time in Table 1 that involves incubations for greater than 3 min. However, it is not possible to relate the data for the activated partial thromboplastin time or prothrombin time (Table 1) to the slower inactivations of factors V and VIII (Figs. 2 and 3). In the former case, activators such as kaolin or tissue factor rapidly generate higher levels of factors Va and VIIIa and the inactivation of these activated cofactors occurs during the clotting assay itself. Neither the rate nor the extent of activation of factors Va or VIIIa in plasma during activated clotting assays is known.

Human factor VIIIa, a highly active form of factor VIII:C that is generated by insolubilized thrombin, is inactivated very rapidly by activated human protein C. The inactivation of untreated factor VIII was biphasic and much slower. The initial phase resembled that of thrombin-activated factor VIII inactivation, whereas the second phase was 30-fold slower than the inactivation of factor VIIIa. Thus, the activated cofactor, factor VIIIa, is inactivated much more rapidly than the untreated cofactor. It has been reported that factor Va is inactivated much more rapidly than factor VIII.

Human protein C can be activated by several proteolytic enzymes, such as the factor X activator of Russell’s viper venom, trypsin, or thrombin. These proteolytic activators also can activate the bovine molecule. Human α-thrombin may be a major activator in vivo. Recently, a cofactor for thrombin-dependent activation of protein C has been found on the surface of endothelial cells of blood vessels. This cofactor accelerates the activation of protein C by four orders of magnitude and suggests that the activation of protein C by thrombin in vivo may occur very rapidly.

Human protein C may provide a very responsive mechanism for antithrombotic regulation. When factors V and VIII are activated by thrombin, their procoagulant activity increases by several orders of
magnitudes. Activated protein C can inactivate these activated cofactors more rapidly than the nonactivated forms of factors V and VIII. Moreover, activated protein C generates profibrinolytic activity when it is infused into dogs. This may reinforce the antithrombotic properties of activated protein C. A pathophysiological relationship among factors V and VIII and protein C was established when it was shown that plasmas from patients suffering inherited combined deficiency of factors V and VIII are missing the inhibitor of activated protein C. It was hypothesized that in these patients there is a chronic consumption of factors V and VIII by trace amounts of activated protein C. The physiologic role of protein C as an important antithrombotic regulatory molecule was recently demonstrated when it was shown that a familial thrombotic disease was associated with an inherited partial deficiency of protein C. Thus, protein C provides one of the major regulatory mechanisms for controlling thrombosis and hemostasis.

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