Identification of Divalent Metal Ion-dependent Inhibition of Activated Protein C by $\alpha_2$-Macroglobulin and $\alpha_2$-Antiplasmin in Blood and Comparisons to Inhibition of Factor Xa, Thrombin, and Plasmin*

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The half-life of activated protein C (APC) was 31 min in citrated blood and 18 min in whole blood. Immunoblotting analysis of citrated blood identified APC-protein C inhibitor (APC-PCI) and APC-$\alpha_2$-antitrypsin complexes. Whole blood contained two additional APC-inhibitor complexes, one stimulated by Ca$^{2+}$ and another by Mg$^{2+}$. The former was identified as APC-$\alpha_2$-macroglobulin (APC-$\alpha_2$M) while the latter was not identified. APC-$\alpha_2$-antiplasmin complexes (APC-$\alpha_2$AP) were identified, comigrating with APC-PCI complexes. Purified $\alpha_2$M and $\alpha_2$AP inhibited APC in the presence of Ca$^{2+}$ ($k_2 = 99$ and 100 $m^{-1} s^{-1}$, respectively. Inhibition of APC and Factor Xa by $\alpha_2$M and inhibition of APC by $\alpha_2$AP was stimulated by Ca$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$. Inhibition of thrombin by $\alpha_2$M and of plasmin by $\alpha_2$AP was not altered by EDTA or Ca$^{2+}$, suggesting divalent metal ions affect APC and Factor Xa rather than the inhibitors. $k_2$ values for the APC inhibitors and their plasma concentrations suggest that PCI and $\alpha_2$-antitrypsin are the more important APC inhibitors and that $\alpha_2$M and $\alpha_2$AP are metal ion-dependent auxiliary inhibitors. Inhibitors can account for the in vivo half-life of APC.

Protein C is a vitamin K-dependent zymogen (1, 2) which, when proteolytically cleaved to form activated protein C (APC), serves as an anticoagulant regulator of coagulation pathways by inactivation of the cofactors Va and VIIIa (3–5). Evidence for its physiologic importance stems from reports of potentially fatal purpura fulminans in homozygous protein C-deficient infants (6) and association in some families of venous thrombotic disease with heterozygous deficiency of protein C (7). Evidence of protein C activation during intravascular coagulation has been presented (8). APC had an anti-thrombotic effect and prevented death due to septic shock when administered in several animal models (9–12). The possibility of future therapeutic use of APC in humans has led to increased interest in its physiologic inhibition by protease inhibitors in blood. Recent work revealed that major inhibitors of APC in plasma include PCI and $\alpha_1$AT (13–16), both of which inhibit APC relatively slowly compared with inhibition of other coagulation enzymes by plasma inhibitors. The existence of other APC inhibitors has been suggested (16). In addition to the two previously identified plasma inhibitors, here we report that $\alpha_2$M, $\alpha_2$AP, and possibly another protein inhibit APC in whole blood in a divalent metal ion-dependent manner, making the half-life of APC in whole blood without divalent metal ion chelators significantly shorter than in citrated blood or plasma. To determine whether the unusual metal ion dependence was due to an effect on APC or to an effect on $\alpha_2$M and $\alpha_2$AP, we compared inhibition of APC to that of Factor Xa, thrombin, and plasmin.

EXPERIMENTAL PROCEDURES

Reagents—Human protein C was purified and activated as described (9). A previously described monoclonal antibody to the light chain of protein C (C3) was used for immunoblotting and for solid phase assay of APC activity (9, 15, 17, 18). This antibody C3 recognizes with equal efficiency protein C, APC, or complexes of APC with PCI or $\alpha_1$AT. For some immunoassays, goat polyclonal antibody to protein C was used (17). Rabbit antibody to PCI was prepared as described (19, 20). Human $\alpha_2$M was a kind gift from Dr. Steven Gonias (University of Virginia Medical School). Human $\alpha_2$AP was obtained from Athens Research and Technology. Recombinant Arg$^{309}$-$\alpha_2$AT was a generous gift from Drs. Michael Courtney and Ranier Bischoff (Transgene, Strasbourg, France). Monoclonal antibody to $\alpha_2$M was obtained from Chemicon. Other antibodies were prepared in rabbits and obtained from Behring. Polyclonal antibody to $\alpha_2$AP was affinity-purified by absorption to $\alpha_2$AP coupled to Affigel 10 (Bio-Rad) according to manufacturer's instructions. The bound antibody was eluted with 3 M NaSCN in Tris-buffered saline (TBS) and immediately dialyzed against TBS. Cephalin and p-nitrophenyl guanidinobenzoate were obtained from Sigma, protein A-Sepharose from Pharmacia LKB, Biotechnology Inc., heparin from Erklin-Simm, and human Factor Xa, thrombin, and plasmin from Enzyme Research Laboratories.

General Methods—Electrophoresis and immunoblotting were performed as previously described (17, 19). The concentrations of $\alpha_2$M and $\alpha_2$AP were determined from absorbance at 280 nm, using $E_{\text{cm}}^{1%}$ of 8.9 (21) and 6.7 (22), respectively. The $\alpha_2$M was determined to be 91% active by titration against active site-titrated thrombin (92% active), and the $\alpha_2$AP was determined to be 67% active by titration against active site-titrated plasmin (14% active), according to procedures previously described (23). The activity of thrombin titrated with $\alpha_2$M was determined by clotting of fibrinogen (Kabi) (24), and the amidolytic activity of plasmin titrated with $\alpha_2$AP was determined using the peptide substrate S-2366 (Kabi).
Kinetics for Inhibition of APC and Other Proteases—For determination of the second order association rate constant ($k_2$) for inhibition of APC by $\alpha_2$M, 0.5 $\mu$M APC was incubated at 37 °C with 2.6 $\mu$M $\alpha_2$M and 5 mM CaCl$_2$ or other additions as specified in TBS containing 0.05% bovine serum albumin. Aliquots of 4 $\mu$L were removed over time, and the residual APC anticoagulant activity was tested by its ability to prolong the activated partial thromboplastin time. A control curve was obtained using dilutions of APC incubated without $\alpha_2$M. Clotting mixtures contained 100 $\mu$L of pooled normal plasma (George King Bio-Medical, Inc.) preincubated 200 s with the test sample and a cephalin-silica activator (100 $\mu$L of Thrombost, Ortho). Clotting was initiated by addition of 100 $\mu$L of 25 mM CaCl$_2$. For inhibition of APC by $\alpha_2$AP, loss of amidolytic activity toward S-2366 was measured over time by assaysing aliquots of mixtures containing APC (63 nM) and $\alpha_2$AP (from 4.6 to 14 $\mu$M). Pseudo-first order rate constants ($k_1$) were determined from the slopes of semilog plots of activity versus time, and $k_2$ was calculated from $k_2 = k_1/b_{[\text{inhibitor}]}$, using the plasma concentration of $\alpha_2$M, $\alpha_2$AP, or other inhibitor.

For determination of the $k_2$ for inhibition of Factor Xa by $\alpha_2$M, 0.18 unit/ml Factor Xa was incubated at 37 °C with 1.12 $\mu$M $\alpha_2$M in TBS, 1% BSA containing 5 mM CaCl$_2$ or other additions as specified in TBS, 1% BSA. Thrombin (22.5 units/ml) was added and incubation continued for selected times from 1 to 3 min. The reaction was quenched by addition of 23 volumes of TBS-BSA, followed by immediate determination of residual Factor Xa activity in a Xa one-stage clotting assay as follows. Normal plasma (100 $\mu$L) was preincubated with 100 $\mu$L of test sample and 100 $\mu$L of 0.2 mg/ml cephalin for 30 min at 37 °C followed by addition of 100 $\mu$L of 50 mM Tris-HCl, 0.1 M NaCl, pH 8.2. The samples were incubated 1 h at 3'7 °C and 2 h at 4 °C. Each sample was then assayed by adding 20 $\mu$L of washed, packed protein A-Sepharose beads in a 1.5-ml conical microcentrifuge tube and mixed. The mixture was incubated 16 h at 4 °C, with occasional mixing during the last hour of incubation. The samples were centrifuged 1 min, and 20 $\mu$L of each supernatant solution was subjected to immunoblotting analysis.

RESULTS

Effect of Metal Ions in Blood on APC Half-life and Inhibitor Complexes—To study the influence of divalent metal ions on the inhibition of APC by blood, APC was mixed with freshly drawn blood in the absence or presence of citrate, and the loss of APC activity was measured as a function of time. As calculated from the APC amidolytic activity data in Fig. 1 (upper panel), the average half-life of APC amidolytic activity in whole blood from seven individuals was 18 ± 2 min, whereas in blood containing 0.01 M trisodium citrate it was 31 ± 5 min ($p < 0.001$). These data were determined in a solid-phase immunocapture microtiter plate assay for APC. Based on direct amidolytic assays of supernatants of centrifuged blood samples in substrate containing a thrombin inhibitor, the respective APC half-life values were 21 and 32 min. Separate experiments in which the blood cells were removed from the incubation mixture immediately after the addition of APC and prostaglandin E-1 gave half-life values in the presence and absence of citrate similar to those obtained in the presence of cells, showing that the shorter half-life in whole blood,

**FIG. 1**. Inhibition and complex formation of APC added to human blood. The upper panel shows inhibition of APC activity over time during incubation with blood with and without the addition of citrate. The mean values from seven samples without citrate from the same donors are indicated by closed circles. Standard errors are indicated by error bars. The microplate assay for APC activity was described under “Experimental Procedures.” The lower panel shows an immunoblot for protein C and APC in blood incubated with APC in the presence or absence of citrate. A representative individual sample from the experiment in the upper panel is shown. The immunoblot was from a 5% nondenaturing polyacrylamide gel and employed monoclonal antibody to protein C (C3) followed by $^{125}$I-protein C (15, 17).
Calcium Ion-dependent Inhibition of Activated Protein C

In the absence of citrate, was not due to any metal ion-dependent reactions involving blood cells under these conditions. Moreover, half-life determinations based on APC anticoagulant activity assays gave 20 min for APC in whole blood without citrate, 33 min in blood with citrate, and in blood from which cells were removed, 17 min without citrate and 28 min in the presence of citrate. Thus, the inhibition of APC anticoagulant activity in blood coincided with loss of amidolytic activity, was significantly influenced by divalent metal ions, and was not significantly influenced by blood cells. Other experiments in which recombiant hirudin (Ciba-Geigy, 2 μg/ml) was included in the blood mixtures suggested that inhibition of APC activity in blood was not influenced by any thrombin that might be present.

The lower panel of Fig. 1 shows an immunoblotting analysis using a nondenaturing gel for protein C antigen in blood samples incubated with APC. When APC was incubated with blood in the presence of citrate (lanes 6–10), there were two apparent major bands of APC-inhibitor complexes formed, similar to previous reports (14, 15) for citrated plasma incubated with APC. Inmunoblotting for PCI or for α-AT (data not shown) confirmed that these bands contained APC-PCI and APC-α-AT, as previously reported (14, 15). However, in the reaction mixtures using whole blood, i.e., without citrate (Fig. 1, lower panel, lanes 1–5), two additional bands containing APC antigen, designated x and y, were visible following incubation of APC with blood. Other experiments (data not shown) demonstrated that addition of recombinant hirudin (2 μg/ml) to the mixtures of blood and APC had no effect on bands x and y or any of the other APC bands.

To investigate whether formation of these bands was divalent metal ion-dependent, metal ions and other additions were made to reaction mixtures of APC with citrated normal plasma or citrated protein S-depleted plasma, and the mixtures were electrophoresed on nondenaturing gels and subjected to immunoblotting for protein C antigen. As seen in Fig. 2 (lanes 1 and 6), the only two apparent bands detected in the absence of added metal ions migrated at positions previously assigned to APC-PCI and APC-α-AT complexes. Addition of 12 mM calcium ions led to the appearance of a new band (band x) of very low electrophoretic mobility (Fig. 2, lanes 2 and 7). Other experiments using protein S-depleted plasma revealed that this low mobility band x seen in Fig. 2 (lane 7) was dependent on calcium ions but not on the presence of protein S. In separate experiments (data not shown), band x formation was also stimulated by magnesium or strontium ions but to a lesser extent than by calcium ions. Addition of a metal ion mixture to give final concentrations of 1.8 mM MgCl₂ and 0.1 mM each ZnCl₂, MnCl₂, CoCl₂, and CuCl₂, led to the appearance of another new band, designated band y (Fig. 2, lane 3). In other experiments appearance of band y was dependent on magnesium ions and not on any of the other divalent ions tested (data not shown). Addition of phospholipids or protein S to citrated plasma did not change the overall pattern of complex formation (Fig. 2, lanes 4 and 5). A diminution of the band ascribed to APC-PCI complexes was observed when calcium ions were added (Fig. 2, compare lanes 2, 3, and 7 with lanes 1 and 6), but not when calcium ions were added in combination with phospholipids (Fig. 2, lanes 4, 5, and 8). When APC was incubated with human serum (Fig. 2, lane 9), bands comigrating with bands x and y were observed. Thus, the divalent metal ions, calcium and magnesium, stimulated the formation of APC complexes in blood, plasma, and serum.

Identification of APC Complexed with α-M and α-AP in Blood—Band x was identified as the complex APC-α-M by its removal from an incubation mixture of blood and APC using monoclonal antibody to α-M, followed by protein A-Sepharose (Fig. 3, compare lanes 3 and 9 with lane 2). Complexes of APC-α-AT were removed in a similar manner by adsorption using anti-α-AT antibodies (Fig. 3, lane 4). Adsorption of reaction mixtures using antibodies to α-antichymotrypsin, C1 inhibitor, inter-α-protease inhibitor, and β₂-glycoprotein I, followed by protein A-Sepharose, did not remove band y or any of the other bands (Fig. 3, lanes 5–8). In other experiments, adsorption using antibodies to plasminogen activator inhibitor-1 did not remove any of the prominent bands containing APC antigen (data not shown).

When the reaction mixtures of blood and APC without citrate were adsorbed using affinity-purified antibody to α-AP followed by protein A-Sepharose, the band previously identified as APC-PCI was diminished (Fig. 3, compare lane 11 with the control in lane 10). Moreover, adsorption using antibody to PCI did not completely remove the band in the region of APC-PCI complexes (Fig. 3, lane 12, compared with lane 10), even though other immunobLOTS developed with antibodies to PCI showed that all detectable complexes containing PCI were present. A diminishing of the band ascribed to α-M and α-AP.
antigen had indeed been removed (data not shown). Control experiments showed that the affinity-purified antibody to \( \alpha_2 \)AP did not recognize PCI (data not shown). Adsorption of the reaction mixture of blood and APC simultaneously using APC-PCI and APC-\( \alpha_2 \)AP complexes were originally present in the immunoblot band labeled APC-PCI (Fig. 2). This was further confirmed using a reaction mixture of PCI-depleted plasma with APC, calcium, and magnesium ions (Fig. 3, lanes 14 and 15). The PCI-depleted plasma formed complexes in the region of APC-PCI (Fig. 3, lane 15), and it was devoid of PCI antigen when analyzed by immunoblotting with antibodies to PCI (data not shown). The identity of these complexes as APC-\( \alpha_2 \)AP was established by their complete removal upon adsorption with affinity-purified antibody to \( \alpha_2 \)AP (Fig. 3, lane 14). Thus, APC complexes with both \( \alpha_2 \)M and \( \alpha_2 \)AP in whole blood.

An additional band containing APC antigen was seen on immunoblots, migrating just below band y in some experiments, e.g., in Fig. 3 (lanes 19 and 12). This band was faint when samples were electrophoresed immediately after incubation of the reaction mixture containing APC, but it increased in intensity when samples were frozen and thawed or handled for extended periods of time prior to immunoblotting analysis, suggesting that this band may arise after proteolysis or degradation of one of the other species containing APC. This band may be related to APC-\( \alpha_2 \)AP, since antibody to \( \alpha_2 \)AP removed this band in Fig. 3, lanes 11 and 13. Moreover, as seen below in Fig. 6, a band of this mobility was detected on immunoblots of plasma/APC mixtures with antibodies against \( \alpha_2 \)AP.

**Inhibition of APC by Purified \( \alpha_2 \)M**—Inhibition of APC by human \( \alpha_2 \)M was studied using purified proteins in the presence of 5 mM calcium ions or 5 mM EDTA. From the slopes of pseudo-first order plots of APC activity against time (Fig. 4, upper panel), the second order association rate constant \( k_2 \) for inhibition of APC anticoagulant activity by \( \alpha_2 \)M in the presence of calcium ions was \( 99 \pm 20 \text{ m}^{-1} \text{s}^{-1} \) (\( n = 3 \)). Inhibition of APC by \( \alpha_2 \)M in the presence of EDTA was negligible. Similar values for \( k_2 \) were obtained by monitoring the inhibition of APC amidolytic activity by \( \alpha_2 \)M; however, there was often a lag phase of up to 30 min before inhibition of APC amidolytic activity by \( \alpha_2 \)M was observed, and maximum inhibition did not exceed 80%. To see whether APC bound to \( \alpha_2 \)M retained some activity against small molecules, but not macromolecules, incubation mixtures of APC and \( \alpha_2 \)M were tested to measure how much of the remaining APC amidolytic activity was inhibited by Arg-Phe-\( \alpha_2 \)AT. After 50 min of incubation of APC with \( \alpha_2 \)M, 26% of the remaining APC amidolytic activity was protected from Arg-Phe-\( \alpha_2 \)AT, after 70 min of incubation, 50% was protected, and after 16 h of incubation, 100% was protected. Thus, APC bound to \( \alpha_2 \)M retained approximately 20% of its amidolytic activity.

Complex formation of APC with purified \( \alpha_2 \)M was directly demonstrated using immunoblotting. Fig. 4 (lower panel) shows an immunoblot for APC antigen from a non-denaturing gel (left) and an immunoblot from a denaturing gel (right) of incubation mixtures of APC with purified human \( \alpha_2 \)M. Bands (Fig. 4, lower left) with the same mobility as band x in Figs. 1 and 2 and APC-\( \alpha_2 \)M in Fig. 3 were apparent after 5 min of incubation in the presence of calcium ions but were barely detectable after 16 h of incubation in the presence of EDTA. The denaturing SDS gel immunoblot revealed a doublet of heat-stable, detergent-stable bands of molecular weight in excess of 200,000 formed after APC was incubated with \( \alpha_2 \)M for \( \geq 5 \) min in the presence of calcium ions. Samples from incubation mixtures of blood and APC immunoblotted from denaturing SDS gels had an identical pattern of immunoblot bands representing complexes of molecular weight over 200,000 (data not shown). The molecular weight of these complexes on reduced denaturing SDS gels was 130,000–150,000 (data not shown). In the non-denaturing gel blot (Fig. 4, lower panel), the total measured radioactivity per lane fell by 33% for the 120-min incubation of APC and \( \alpha_2 \)M in the presence of calcium ions, but the radioactivity per lane remained constant during 120 min of incubation of APC in the presence of calcium ions with \( \alpha_2 \)M and remained constant during 120 min of incubation of APC and \( \alpha_2 \)M in the presence of EDTA. This indicates that APC in the APC-\( \alpha_2 \)M complexes was under-represented in the immunoblots, either because APC was not fully accessible to the antibody or because the large APC-\( \alpha_2 \)M complexes did not transfer efficiently to the nitrocellulose paper, or both. In the denaturing SDS gel blot (Fig. 4, lower panel), the total radioactivity per lane also decreased for the 30-min incubation with APC and \( \alpha_2 \)M in the presence of calcium ions, but not in the presence of EDTA. Thus, although immunoblotting analysis qualitatively demonstrates complexation of APC with purified \( \alpha_2 \)M, it does not allow a quantitation of these complexes.

To learn whether the unusual metal ion dependence of inhibition of APC by \( \alpha_2 \)M might be due to APC or to \( \alpha_2 \)M, \( k_2 \) values for inhibition by \( \alpha_2 \)M of APC, Factor Xa, and thrombin were obtained using kinetic studies and then compared under various conditions. The data in Table I show that 5 mM CaCl\(_2\) stimulated APC inhibition at least 7-fold and Factor Xa inhibition 4-fold when compared with 0.2 mM EDTA. Fig. 5 shows that the \( k_2 \) for inhibition of APC had a similar dependence on calcium ion concentration as the \( k_2 \) for inhibition of the homologous vitamin K-dependent Factor Xa. The \( k_2 \) values obtained for inhibition of Factor Xa are in reasonable agreement with those previously reported (25), which were...
TABLE I
Second order rate constant ($k_2$) for inhibition of proteases by $\alpha_2M$ (M$^{-1}$ s$^{-1}$)

| Addition          | APC$^a$ | Factor Xa$^a$ | Thrombin$^a$ |
|-------------------|---------|---------------|--------------|
| Citrate (11 mM)   | 22 ± 15 | 343 ± 140     | 6900 ± 800   |
| EDTA (0.2 mM)     | <12     | 190 ± 68      | 7800 ± 1200  |
| TBS               | 30 ± 18 | 480 ± 21      | 7100 ± 1600  |
| CaCl$_2$ (5.0 mM) | 88 ± 14 | 770 ± 240     | 7200 ± 1500  |
| MgCl$_2$ (5.0 mM) | 70 ± 19 | 643 ± 46      | ND           |
| MnCl$_2$ (0.5 mM) | 63 ± 3  | 2100 ± 420    | ND           |
| MnCl$_2$ (0.5 mM) | 83 ± 24 | 910 ± 230     | ND           |

$^a$ Values for inhibition of APC or Factor Xa are the means of three or more determinations ± standard deviations. Values for inhibition of thrombin are the means of five determinations ± standard deviations. ND, not determined.

To determine whether this unusual metal ion dependence of inhibition of APC by $\alpha_2$AP was due to APC or to $\alpha_2$AP, inhibition of APC was compared with that of plasmin by $\alpha_2$AP. The data in Table II show that inhibition of APC by $\alpha_2$AP was 4-fold more effective than magnesium ions in stimulating this inhibition of APC. Heparin at 1 unit/ml diminished inhibition of APC by $\alpha_2$AP. Kinetic analyses showed that the association rate constant $k_2$ for inhibition of APC by $\alpha_2$AP was $100 \pm 9$ M$^{-1}$ s$^{-1}$ in the presence of calcium and magnesium ions and 28 M$^{-1}$ s$^{-1}$ in the presence of EDTA.

The quantity of APC-azAP complexes in the 1-h incubation mixtures shown in Fig. 7 (right panel) did not appear to be significantly different in the presence or absence of divalent metal ions. However, in other experiments (not shown) divalent metal ions stimulated the formation of these complexes at incubation times of less than 30 min. Thus, purified $\alpha_2$AP inhibits APC and forms complexes identifiable on immunoblots of reaction mixtures of APC with the purified inhibitor or plasma. Immunoblots for PCI antigen (not shown)
FIG. 7. Immunoblots for purified APC-α2AP and for APC-α2AP in various plasmas. Pooled normal plasma (NHP), PCI-depleted plasma (PCLd), or α2AP-congenitally deficient plasma (α2APd) were incubated for 1 h alone, with APC (+APC), or with APC and metal ions (+Me). The metal salts used were 6.5 mM CaCl₂ and 1.8 mM MgCl₂, and the final APC concentration was 4 μg/ml. The samples were subjected to electrophoresis on a 5% nondenaturing gel and then immunoblotted for APC (left panel) or for α2AP (right panel). The last lane of each blot contains complexes of APC-α2AP formed by incubating purified α2AP at 90 μg/ml with APC at 140 μg/ml in TBS, 1% BSA in the presence of 5 mM CaCl₂ and 1.8 mM MgCl₂ at 37 °C for 6 h. The last lane in the left blot contains, in addition, complexes of purified APC-PCI formed in a similar manner.

**DISCUSSION**

Inhibition of APC by the plasma inhibitors α1AT and PCI is not divalent metal ion-dependent in purified systems or in plasma (14, 20, 28), and the inhibition of plasma blood coagulation enzymes by plasma protease inhibitors has not been previously shown to be stimulated by divalent metal ions. However, as described here, we found a difference in the half-life of APC in whole blood in the presence and absence of citrate, a divalent metal ion chelator, and this led us to discover the existence of two additional types of apparent APC-inhibitor complexes that are observed in blood or serum when citrate is absent. One of these complexes is identified here as APC-α2M, and the other one has not yet been identified or indeed proven to be associated with inhibition of APC. In addition, divalent metal ion-stimulated complexes of APC-α2AP are shown here to form when APC is added to plasma, blood, or serum, and these complexes comigrate on immunoblots with APC-PCI complexes.

The immunoblotting observations prompted kinetic studies using purified proteins. Purified human α2M and α2AP inhibit APC in calcium ion-stimulated reactions with second order association rate constants of 99 and 100 M⁻¹ s⁻¹, respectively, compared with 11 M⁻¹ s⁻¹ for inhibition of APC by α1AT and 6.0 × 10⁸ M⁻¹ s⁻¹ for inhibition of APC by PCI (14, 20, 28). Based on the plasma concentrations for α2M (3 μM), α2AP (1 μM), α1AT (40 μM), and PCI (88 nM), the calculated half-life of APC in blood considering each inhibitor separately is 38, 110, 26, and 22 min, respectively. This kinetic estimate of relative reactivity of inhibitors in plasma is consistent with the suggestion from the immunoblotting data that α1AT and PCI are primary inhibitors, whereas α2M and α2AP are auxiliary inhibitors of APC in blood. The observed half-life of APC in whole human blood without citrate of 18–21 min is approximately what the combination of calculated half-lives would imply and is similar to the reported half-life of 23 min for APC infused into humans (29) and is slightly longer than the in vivo half-life of 10–14 min for APC infused into baboons (9). The discovery of divalent metal ion-dependent inhibition of APC by α2M, α2AP, and possibly by one other inhibitor in blood helps to explain the differences between the in vivo half-life of APC and the in vitro half-life of 31 min in citrated plasma. Thus, inhibition of APC by protease inhibitors in blood could entirely account for the regulation and removal of APC activity in vivo.

APC has approximately nine calcium ion binding sites (30), and calcium ions and phospholipids are required for optimal rates of inactivation of Factors Va and VIIIa (3, 5). Most of the calcium ion binding sites reside in the NH₂-terminal domain containing nine γ-carboxyglutamyl (Gla) residues, but APC with the Gla domain removed contains at least one high affinity binding site for calcium or manganese ions that may alter APC conformation (30, 31). Calcium ions are not required for inhibition of APC by PCI (20), α1AT (14, 28), or plasminogen activator inhibitor-1 (32) or for inhibition of other coagulation serine proteases by plasma protease inhibitors. Consequently, finding a strong influence of calcium ions on inhibition of APC by α2M and α2AP was unexpected.

To assess whether the divalent metal ion influences either the enzyme or the protease inhibitor in these reactions, the inhibition of Factor Xa and thrombin by α2M and of plasmin by α2AP was studied. Inhibition of the vitamin K-dependent homologue, Factor Xa, is stimulated by divalent metal ions, whereas inhibition of thrombin by α2M or of plasmin by α2AP is not divalent metal ion-dependent. Thus, the unusual divalent metal ion dependence of inhibition of APC and Factor Xa is probably due to a property of the enzymes rather than due to a property of the inhibitors, α2M and α2AP.

α2M, a tetramer of subunits of M, 180,000, inhibits a wide variety of proteases by a mechanism in which the protease is entrapped in a cataglike structure (33, 34). The protease active site is not altered, and it may still exhibit partial enzymatic activity toward small substrates. The protease usually leaves a “bait” region of the α2M, causing a change in conformation which stimulates covalent attachment between reactive thioester groups of α2M and NH₂ groups of the protease and which closes the cage. Such a mechanism for α2M and APC interactions is consistent with the observation here that APC amidolytic activity is diminished but cannot be entirely inhibited and that, over time, the remaining observed amidolytic activity becomes less susceptible to inhibition by Arg10³α1AT, a very efficient macromolecular inhibitor that neutralizes >98% of APC (28). APC in APC-α2M complexes is probably less accessible to anti-protein C antibodies as well, since the total detectable anti-protein C antibody per lane on immunoblots decreased as APC-α2M complexes increased in incubation mixtures of APC and α2M. Since APC-α2M complexes on immunoblots were stable to heat and detergent treatment of samples taken after 5 min of incubation of APC with blood or with purified α2M, it appears that some of the APC-α2M complexes are covalent. In fact, the APC-α2M complexes were equally intense on denaturing SDS gel immunoblots as on non-denaturing gel immunoblots. The apparent molecular weight of APC-α2M complexes on reduced SDS gels of 130,000–150,000 suggests that the light or the heavy chain of APC is linked to fragments of α2M in the range of M, 90,000–120,000. Such fragment sizes have been reported for cleaved α2M (35), suggesting that APC cleaves α2M. α2M inhibits thrombin (26) and Factor Xa (25, 36); however, we are not aware of any previous reports of divalent metal ion-dependent inhibition by α2M of these or any other proteases. Investigators previously found that only about 13% of ¹²⁵I-Factor Xa incubated with mouse or human citrated plasma bound to α2M, whereas 90% of ¹²⁵I-Factor Xa infused into mice was...
bound to α2M within 2 min (36). The difference between these in vitro and in vivo data could be explained as due to divalent metal ion stimulation of the inhibition of Factor Xa by α2M, as seen here. It was reported that purified α2M and α2M in plasma do not inhibit APC, but the experiments described by these authors (37) apparently did not include the addition of divalent metal ions to reaction mixtures. Perhaps the divalent metal ion-dependent conformation of APC can fit into the cagelike structure of α2M in a manner to cleave the bait region, or perhaps APC in this conformation is more reactive with some macromolecular substrates.

α2AP, a glycoprotein of M, 70,000, is a member of the serpin superfamily (38, 39) and is homologous to PCI and α1AT. It interacts very efficiently with plasmin with a k0 of 4 × 107 M⁻¹s⁻¹, forming 1:1 complexes that are predominantly covalent. Inhibition of APC by α2AP is stimulated by divalent metal ions in a purified system and in blood. Interestingly, after 30 min of incubation of plasma with APC, complexes observed on immunoblots are more intense in the absence of divalent metal ions than in their presence (Fig. 6, and data not shown). Proteolysis of α2AP or its complexes in plasma containing divalent metal ions may be responsible for this observation. We are unaware of previous reports of divalent metal ion stimulation of inhibition of proteases by α2AP.

Complexes of APC with α1AT and PCI were identified in plasma of patients with disseminated intravascular coagulation (8), in baboons infused with APC (40), and in chimpanzees infused with phospholipid vesicles containing Factor Xa (41). In the latter report, additional APC complexes were detected on immunoblots but not identified. We have recently identified APC-α2M and APC-α2AP complexes in plasma of baboons infused with APC (42). Thus, α1AT, PCI, α2M, and α2AP each complex in vivo with infused APC, and each inhibitor can function physiologically to neutralize APC.

In summary, divalent metal ions in blood significantly enhance the rate of inhibition of APC by the protease inhibitors α2M and α2AP, and the inhibition of APC by protease inhibitors in blood may adequately account for the clearance and in vivo half-life of APC.

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