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ZINC IONS BIND TO AND INHIBIT ACTIVATED PROTEIN C

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Running title: Zn^{2+} inhibits activated protein C

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Summary

Zn^{2+} ions were found to efficiently inhibit activated protein C (APC), suggesting a potential regulatory function for such inhibition. APC activity assays employing a chromogenic peptide substrate demonstrated that the inhibition was reversible and the apparent K_I was 13 ± 2 μM. k_{cat} was 7-fold decreased whereas K_M was unaffected in the presence of 10 μM Zn^{2+}. The inhibitory effect of Zn^{2+} on APC activity was also observed when factor Va was used as a substrate in an assay coupled to a prothrombinase assay. The interaction of Zn^{2+} with APC was accompanied by a reversible ~40% decrease in tryptophan fluorescence, consistent with the ion inducing a conformational change in the protein. The apparent K_D was 7.4 ± 1.5 μM and thus correlated well with the apparent K_I. In the presence of physiological Ca^{2+} concentration the K_I and K_D values were 3–4-fold enhanced, presumably due to the Ca^{2+}-induced conformational change affecting the conformation of the Zn^{2+}-binding site. The inhibition mechanism was non-competitive both in the absence and presence of Ca^{2+}. Comparisons of sequences and structures suggested several possible sites for zinc binding. The magnitude of the apparent K_I in relation to the blood and platelet concentrations of Zn^{2+} supports a physiological role for this ion in the regulation of anticoagulant activity of APC. These findings broaden the understanding of this versatile serine protease and enable the future development of potentially more efficient anticoagulant APC variants for treatments of thrombotic diseases.

Key words

Activated protein C, Serine protease, Zinc, Blood coagulation, Enzyme inhibition

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Introduction

Activated protein C (APC) is a ~60 kDa blood plasma glycoprotein, which functions as an important vitamin K-dependent serine protease down-regulating blood coagulation (1). The structure consists of four domains, an N-terminal γ-carboxyglutamic acid (Gla) domain that contains nine carboxylated glutamic acids, two epidermal growth factor-like regions (EGF-1 and EGF-2) and a serine protease domain containing the catalytic site residues His57, Asp102 and Ser195 (chymotrypsin numbering) (2). Protein C circulates in blood as an inactive zymogen that is activated via proteolytic cleavage by thrombin bound to thrombomodulin in the presence of the endothelial protein C receptor (EPCR) (3). APC down-regulates coagulation by degrading coagulation factors Va and VIIIa, the cofactors for factors Xa and IXa, respectively, in enzyme complexes on phospholipid surfaces in the presence of factor S (2, 4). In addition to the anticoagulant properties, APC has anti-inflammatory, anti-apoptotic and cytoprotective effects, which are dependent on the APC-mediated cleavage of the protease-activated receptor PAR-1 in the presence of EPCR, leading to a number of signaling events (1, 3, 5, 6).

The activity of APC is modulated by Ca^{2+}. The nine carboxylated glutamates in the Gla domain are able to bind to Ca^{2+}, which changes the conformation of this domain and thus allows both the inactive and active forms of protein C to bind to phospholipid membranes. Interactions with phospholipids are required for the enzyme complexes catalyzing the activation of protein C as well as for those in which APC exerts its proteolytic functions. In addition to the Gla domain, APC has one Ca^{2+}-binding site in the EGF-1 domain and one in the 70-80-loop in the serine protease domain (7-9). The binding of Ca^{2+} to EGF-1 is required for the biological activity of protein C, and it has been proposed that it is involved in the interaction of the enzyme with other proteins, including factor S (9). Binding of Ca^{2+} to the

70-80-loop is accompanied by a conformational change that leads to an ~6% decrease in

tryptophan fluorescence. This interaction is required for the activation of protein C by the

thrombin-thrombomodulin complex, and it also somewhat stabilizes the amidolytic activity

of APC (8, 10). A similar enhancement on the amidolytic activity by Mn^{2+} ions has been

observed. This effect is believed to be mediated via the Ca^{2+} binding site in the serine

protease domain of APC (11). The amidolytic activity of APC is also modulated by Na^+ and

it has been suggested that the 221-225 loop in the serine protease domain forms a binding site

for this ion (12). A previous report showing that Gla domain-less APC requires Na^+ for its

amidolytic activity in the absence but not in the presence of Ca^{2+}, suggested that an allosteric

link between the Na^+- and Ca^{2+}-binding loops modulates the structure and function of APC

(13).

No metal ions have previously been reported to down-regulate the activity of APC. In

the present investigation we found that Zn^{2+} efficiently inhibits the activity of APC, with both

a synthetic peptide and factor Va as substrates. We have therefore characterized the

interaction of Zn^{2+} with the enzyme, and its influence on the kinetics of the amidolytic

reaction. Zn^{2+} binding was accompanied by a 40% decrease in tryptophan fluorescence,

which was used to determine the affinity of APC for Zn^{2+}. Structural comparisons and other

data are used to propose reasonable models for how the inhibition might arise.

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Materials and methods

Proteins

Human full-length APC and APC lacking the Gla-domain (Des-Gla APC) were purchased from Enzyme Research Laboratories (South Bend, IN, USA). The preparations were >90 % homogenous in 10 % SDS-PAGE under reducing conditions with the Laemmli system. Both the major and minor bands were subjected to peptide mapping by Maldi-Tof MS as described before (14), verifying that all bands were APC. The active concentrations of the APC preparations, determined by stoichiometric titrations against protein C inhibitor (PCI) with a known active concentration (14, 15), were used throughout this study. Human protein S, human α-thrombin, human factor Xa, human prothrombin and bovine factor V/Va were purchased from Enzyme Research Laboratories (South Bend, IN, USA). The active concentrations of factor Xa and α-thrombin were determined as described previously (14, 16).

Experimental conditions

Enzyme assays and fluorescence measurements were conducted at 25±0.2°C and pH 7.4 in 20 mM Tris-HCl buffer containing 0.1 M NaCl, 0.1 % PEG 8000, except assays containing phospholipid vesicles, for which the PEG was excluded. If indicated, various concentrations of EDTA or a divalent cation as a chloride or sulfate salt were added. The salts used were PA grade from Merck. Unless noted otherwise, all Zn^{2+} was added as $ZnCl_2$. The water used in buffers had first been purified by the central water purification system at the Uppsala Biomedical Center, followed by purification in a Milli-Q instrument (Millipore), and had a resistance of 18.2 mega Ohm/cm.

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Chromogenic assays for APC, thrombin and factor Xa activity

The amidolytic activity of APC was measured spectrophotometrically in a Hitachi U-2000 dual-beam spectrophotometer with the chromogenic peptide substrate L-pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline hydrochloride (S-2366 from Haemochrom Diagnostica, Mölndal, Sweden). The increase in absorbance due to product formation was monitored for 60 seconds at 405 nm. For APC activity, all preincubations were done with at least 100 nM APC, a concentration at which the enzyme was stable under the conditions used. The enzyme was then diluted to 1-2 nM in buffer containing S-2366 at the start of the measurement. The activities of thrombin and factor Xa were measured by similar assays but with the use of the chromogenic peptide substrates S-2238 and S-2222, respectively (Haemochrom Diagnostica, Mölndal, Sweden), as described before (14, 16).

Kinetics of APC activity in the presence of Zn^{2+}

The effects of Zn^{2+} on K_M and k_{cat} for the amidolytic activity of APC were determined by measuring APC activity by the chromogenic assay in the presence of either 10 μM Zn^{2+}, 100 μM EDTA, 2.5 mM Ca^{2+} or 2.5 mM Ca^{2+} and 15 μM Zn^{2+} at S-2366 substrate concentrations varying from 0-3 mM. The somewhat higher zinc concentration used in the presence than in the absence of Ca^{2+} was choosen due to the K_D for Zn^{2+} being somewhat higher in the presence than in the absence of Ca^{2+} (see below). Zn^{2+} was added to APC at least 2 min before each measurement to ensure that equilibrium was reached. The data were analyzed by nonlinear regression fitting to the Michaelis-Menten equation to give K_M and k_{cat}.

The apparent inhibition constant, K_I, for the inhibition of APC by the ion was measured at Zn^{2+} concentrations varying from 0-100 μM and a constant substrate concentration of 0.5 mM. Zn^{2+} was added to APC or des-Gla APC at least 2 min before each measurement. The determination was done both in the absence and presence of 2.5 mM Ca^{2+}

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for full-length APC and in the absence of Ca^{2+} for des-GLA APC. The rate of substrate

hydrolysis was plotted versus the concentration of Zn^{2+}. K_I was obtained by nonlinear

regression fitting of this plot to the equation for non-competitive inhibition (Eq. 1) (17).

$$v_i = K_I \times v_0 /(K_I + I) \quad \text{(Eq. 1)}$$

where v_i is the initial enzymatic rate in the presence of inhibitor, v_0 is the initial enzymatic

rate in the absence of inhibitor and [I] is the concentration of the inhibitor, i.e. Zn^{2+}.

Inactivation of factor Va by APC

The effect of Zn^{2+} on the activity of APC was also determined with factor Va as a

substrate. The time course and the Zn^{2+} concentration dependence for the inactivation of

factor Va was measured by a two-stage assay, essentially as described previously (18). In the

first stage, factor Va (2.4 nM) was incubated with APC (0.2 nM) on 25 µM PC/PS vesicles in

buffer containing 2.5 mM Ca^{2+} in the presence or absence of 100 µM Zn^{2+} for 1-12 min.

Alternatively the time of incubation was fixed at 2 min and the Zn^{2+} concentration varied

from 0 to 100 µM, either in the absence or presence of 0.2 nM protein S. In the second stage,

the remaining factor Va activity was determined in a prothrombinase assay, in which the

factor Va-enhanced rate of prothrombin activation by factor Xa was determined. The

incubation mixture from the first stage was diluted 24-fold into the prothrombinase assay,

which was carried out for 15 min with 25 µM PC/PS vesicles, 0.6 µM prothrombin and 1 nM

factor Xa at 25°C. The remaining activity of factor Va was determined from the decrease of

the rate of thrombin generation, as monitored by an amidolytic activity assay with 200 µM S-

2238, with the absorbance measured at 405 nm in an ELISA reader (Infinite M200 from

Tecan or SpectraMax Plus[384] from MDS Analytical Technologies). All values are the average

of at least three independent measurements ± standard error.

Fluorescence spectra and fluorescence titrations

Fluorescence spectra and titrations were measured in a volume of 500 μl in 0.5×0.5 cm

quartz cuvettes with magnetic stirring in an SLM 4800S spectrofluorimeter, modernized with

Olis electronics and software (Bogart, GA). All fluorescence values reported were corrected

for dilution and inner filter effects.

The effect of the interaction of Zn^{2+} on the fluorescence of APC was analysed by

measurements of fluorescence spectra for APC and des-Gla APC. The optimal λ_{ex} was first

determined to be 282 nm by excitation spectra. Emission spectra were subsequently measured

from λ_{em} 300 to 450 nm at the optimal λ_{ex} with 2 and 4 nm excitation and emission

bandwidths, respectively. The spectra were measured at 100-200 nM protein concentrations.

Equilibrium binding was studied by fluorescence titrations monitored by the decrease

in tryptophan fluorescence accompanying the interaction of Zn^{2+} with APC. Excitation and

emission wavelengths of 282 and 345 nm, respectively, both experimentally determined to

give the highest fluorescence, and excitation and emission bandwidths of 2 and 16 nm,

respectively, were used. APC concentrations varied between 100 and 500 nM. K_D values

were determined by fitting the titration data to the equilibrium binding equation by nonlinear

least-squares analysis, as described before, assuming a 1:1 binding stoichiometry (15).

Modeling of Zn ion into the catalytic module

Sequences similar to that of APC were located among the protein entries of GenBank (19)

and aligned using CLUSTAL W (20). All similar catalytic domain structures were obtained

from the Protein Data Bank (PDB) (21), then superimposed and compared with the programs

LSQMAN (22) and O (23). O was used to model the zinc ions and the structural figures were

prepared using O and Molray (24).

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Results

Effects of divalent cations on APC amidolytic activity

The amidolytic activity of APC was measured spectrophotometrically with the use of the

chromogenic peptide substrate S-2366 (0.5 mM) in the presence of either EDTA or one of the

metal salts $MnCl_2$, $MgCl_2$, $NiCl_2$, $CuSO_4$, and $ZnCl_2$ (Fig. 1). The effects of all the salts were

determined at two concentrations, one corresponding to the physiological concentration of

each metal ion in blood (25-28) and one which was the same for all the salts, i.e. 2.5 mM.

Three of the salts, $NiCl_2$, $CuSO_4$ and $ZnCl_2$, lowered APC activity in a concentration-

dependent manner. Since Cu^{2+}, Ni^{2+} and Zn^{2+} ions have similar sizes (~0.7–0.75 Å), the same

charge and usually similar ligands and coordination, they presumably inhibit APC by similar

mechanisms. At physiological concentrations Zn^{2+} was, however, a more efficient inhibitor

than Cu^{2+} and Ni^{2+} and reduced APC activity by ~60%. Zn^{2+} was chosen for further

experiments not only because of this efficiency but also because, in contrast to copper ions, it

is not redox reactive, simplifying the interpretation of the data. It should be noted, however,

that Cu^{2+}, besides Zn^{2+}, also may have a regulatory effect on APC activity. A similar

inhibiting effect was observed when $ZnSO_4$ was used instead of $ZnCl_2$ (not shown), verifying

that the metal ion was the inhibiting component in both salts.

Effects of Zn^{2+} on APC cleavage of S-2366

The amidolytic activity of APC was studied at different substrate concentrations in the

presence of either 100 μM EDTA, 10 μM $ZnCl_2$, 2.5 mM Ca^{2+} or 2.5 mM Ca^{2+} and 15 μM

Zn^{2+}. The Zn^{2+} concentrations used are in the range of the total physiological Zn^{2+}

concentrations in blood. The plots of APC activity versus the substrate concentration fit well

to the Michaelis-Menten equation (Fig. 2A). The K_M value determined in the presence of

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Zn^{2+} was somewhat lower than that determined in the presence of EDTA (Table 1),

demonstrating that the binding of Zn^{2+} does not decrease the affinity of APC for the

substrate. In contrast, k_{cat} was ~7-fold decreased, demonstrating that Zn^{2+} dramatically

inhibits the mechanism by which APC cleaves the substrate. In the presence of 2.5 mM Ca^{2+},

15 μM Zn^{2+} did not induce any significant effect on K_M but reduced k_{cat} ~2-fold (Table 1).

Thus, Zn^{2+} inhibited APC by decreasing k_{cat} both in the absence and presence of Ca^{2+}.

The amidolytic activities of APC and des-Gla APC were also measured at various

concentrations of $ZnCl_2$ with 0.5 mM substrate. The plots of the decrease in APC activity

versus the increase in $[Zn^{2+}]$ fit well to the equation for non-competitive inhibition (Eq. 1)

(Fig. 2B) giving K_I values of 13 ± 2 and 25 ± 1 μM for APC and des-Gla APC, respectively

in the absence of Ca^{2+} (Table 1). These results demonstrate that the Gla-domain is not of

major importance for the inhibitory effect of Zn^{2+} on APC activity. When the amidolytic

activity was measured in the presence of 2.5 mM Ca^{2+}, the K_I value for zinc inhibition of

APC was 56 ± 4 μM. At saturating concentration of Zn^{2+}, the APC activity was completely

abolished.

To compare the effect of Zn^{2+} on APC activity with that on the activity of

procoagulant proteases whose cofactors APC degrades, thrombin and factor Xa activities in

the presence of Zn^{2+} were also measured. Only a slight decrease in the activities of these

proteases was observed in the presence of Zn^{2+}. The decreases in thrombin and factor Xa

activities were ~40 % at 2.5 mM Zn^{2+}, corresponding to K_I values of over 5 mM. The

concentrations of Zn^{2+} required for the inhibition of thrombin and factor Xa are thus at least

two orders of magnitude higher than those required for the inhibition of APC.

The reversibility of the inhibitory effect of Zn^{2+} on APC activity was determined by

adding 20 μM EDTA to an APC solution containing 10 μM Zn^{2+}. After the addition of 10

μM Zn^{2+}, the activity was 59 ± 1 % of the control value and after further addition of EDTA,

the activity was fully recovered (103 ± 7 % of the control), where the control was APC

containing 20 μM EDTA. The results thus showed that the inhibitory effect of Zn^{2+} on APC

activity was fully reversible.

Effects of Zn^{2+} on the inactivation of factor Va by APC

The effects of zinc ions on the inactivation of factor Va by APC were determined by a two-

stage assay. In the first stage, factor Va was incubated with APC in the presence of

phospholipid vesicles and physiological Ca^{2+} concentration for various time-points with or

without 100 μM Zn^{2+}, followed by a prothrombinase assay in the second stage. The zinc

concentration used was calculated to result in ~83% saturation of the APC (0.2 nM) used in

this assay, based on the apparent affinity of APC for Zn^{2+} in the presence of Ca^{2+}. The

activity in the prothrombinase assay as a function of the time of incubation of factor Va and

APC in the absence or presence of Zn^{2+} is shown in Figure 3A. The activity decreased much

more rapidly in the absence than in the presence of Zn^{2+}, demonstrating that this ion

significantly lowered the ability of APC to inactivate factor Va.

The influence of the Zn^{2+} concentration on the inactivation of factor Va was studied

by a similar two-stage assay as described above but with varying concentrations of Zn^{2+} and a

fixed incubation time of 2 min. This experiment was done in the absence or presence of

equimolar concentration of the cofactor for APC, protein S. In the absence of factor S, the

amount of thrombin generated, reflecting the inhibition of APC activity, increased dose-

dependently in a hyperbolic manner from 0-100 μM Zn^{2+} (Fig. 3B). The inhibitory effect of

zinc ions was also observed in the presence of the cofactor, although with a shift toward

somewhat higher Zn^{2+} concentrations and a different shape of the Zn^{2+}-concentration

dependency. This finding may be partially explained by the previous observation that

commercial protein S preparations contain very little Zn^{2+}, in contrast to human protein S

purified by immunoaffinity chromatography, which contains the Zn^{2+}-bound form of the

protein (29). A small portion of the Zn^{2+} used in our assay may thus have been consumed by

protein S to retain the Zn^{2+}-bound form of the protein. The concentration of protein S was,

however, very small compared to that of Zn^{2+}. Another potential explanation may thus be that

the Zn^{2+}-binding site is slightly less exposed when APC is bound to protein S.

Effects of Zn^{2+} on tryptophan fluorescence spectra

The effect of the interaction with Zn^{2+} on the fluorescence of APC and des-Gla APC was

assessed by measurements of tryptophan fluorescence emission spectra for APC and des-Gla

APC (Fig. 4A & B). The maximal emission was at 345 nm for both proteins. The addition of

500 μM Zn^{2+} to 200 nM APC, calculated to give ~98% saturation based on the K_D

determined below, resulted in ~40 and 30% decreases in the fluorescence intensity at 345 nm,

for APC and des-Gla APC, respectively. The wavelength of the fluorescence emission

maximum was not affected by the interaction with Zn^{2+}.

The reversibility of Zn^{2+}-binding to APC was evaluated by measuring tryptophan

fluorescence emission spectra before and after the addition of 200 μM EDTA to 200 nM

APC containing 100 μM $ZnCl_2$ (Fig. 4C). These concentrations of APC and Zn^{2+} were

estimated to result in ~93% of saturation of APC. The addition of 200 μM EDTA to APC in

the absence of Zn^{2+} resulted in a small decrease in fluorescence, presumably due to EDTA

quenching the tryptophan fluorescence of APC. The addition of 100 μM $ZnCl_2$ to 200 nM

APC lowered the fluorescence of APC, as estimated, and after further addition of 200 μM

EDTA to this solution, the fluorescence was restored to that of APC containing EDTA but

not Zn^{2+} (Fig. 4C). These findings show that the decrease in fluorescence caused by the

interaction of Zn^{2+} with APC is fully reversible. Similar results were obtained when the

reversibility of the Zn^{2+}-induced decrease in fluorescence of des-GLA APC was studied.

Emission spectra for APC measured in the presence or absence of Ca^{2+} showed a ~6%

decrease in fluorescence at saturating conditions, in agreement with previous reports (8, 30).

Attempts to restore the 40% Zn^{2+}-induced decrease in fluorescence by adding excessive

amounts of Ca^{2+} ions were not successful, indicating that Zn^{2+} binds to a site distinct from the

Ca^{2+}-binding sites on APC.

Binding affinities determined by fluorescence

The decrease in fluorescence caused by Zn^{2+} binding to APC was used to determine apparent

K_D values from fluorescence titration curves. The relatively weak binding affinity precluded

assessment of binding stoichiometry. The data were well fitted to the equilibrium binding

equation (15) with the assumption of an equimolar binding stoichiometry (Fig. 5). K_D values

were 7.4±1.5 and 19±3 μM (Table 1) for the interactions of Zn^{2+} with APC and des-Gla APC,

respectively, in the absence of Ca^{2+}. In the presence of 2.5 mM Ca^{2+} the K_D value was 20±3

μM for the interaction of Zn^{2+} with APC.

Discussion

An initial finding in our laboratory that the amidolytic activity of APC was abolished in the

presence of Zn^{2+} initiated this investigation, in which we have characterized the interaction of

Zn^{2+} with APC and quantified the inhibitory effect of Zn^{2+} on APC activity. The unaffected

or somewhat decreased K_M and 7-fold decreased k_{cat}, determined by varying the

concentrations of a chromogenic peptide substrate at a zinc concentration of 10 μM,

demonstrated that Zn^{2+} inhibited APC by a non-competitive mechanism. These results

suggest that metal binds at a position distinct from the active site and that a conformational

change of APC is involved in generating a catalytically inert form of the protease. Such a

conformational change was also supported by the ~40% decrease in tryptophan fluorescence

induced by the binding of Zn^{2+} to APC. The agreement between the K_D determined by

fluorescence and the K_I determined by a chromogenic assay, support that Zn^{2+}-binding results

in the inhibition of the enzymatic activity. The 3–4-fold higher K_D and K_I values in the

presence than in the absence of physiological Ca^{2+} concentration showed that Ca^{2+} affected

the affinity of APC for Zn^{2+}. Nevertheless the inhibition of APC by Zn^{2+} was still non-

competitive in the presence of Ca^{2+}, as demonstrated by the unaffected K_M and decreased k_{cat}.

The catalytic machinery was inhibited by zinc ions both using a chromogenic peptide

substrate, and in a more physiological assay based on the degradation of factor Va by APC in

the presence of phospholipid vesicles and physiological Ca^{2+} concentration. These results

thus support a role of zinc ions in regulating the anticoagulant activity of APC.

The K_I in the low micromolar range, comparable to the concentrations of zinc ions

found in blood plasma and platelets, suggests that the inhibition of APC by these ions may be

physiologically relevant. The total concentration of zinc ions in blood plasma is ~15–20 μM,

whereas the concentration of free zinc is only around 0.15–0.5 μM, because the majority of

these ions in blood are bound to proteins (31-33). Zinc ions are, however, enriched in

platelets at a total concentration of approximately 500 μM, distributed between the α-

granules and cytoplasm (33). Several previous studies have shown that zinc ions are released

during platelet activation and it has been proposed that such release leads to a local increase

in the concentration of Zn^{2+} at the platelet surface (34-37). The exact concentration of Zn^{2+} at

this surface is difficult to estimate. Blood coagulation is, however, known to be very local

and restrictive. Since the surface of the activated platelet is a potential site of action of APC,

due to the presence of factors Va and VIIIa of the tenase and prothrombinase complexes,

respectively, it is highly possible that the activity of APC is down-regulated by Zn^{2+} on this

surface. The Zn^{2+}-concentration dependence for the inactivation of factor Va by APC

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determined in this study was clearly dose-dependent in a hyperbolic manner from 0-100 μM Zn^{2+}, but was somewhat shifted to higher Zn^{2+} concentration in the presence of factor S. This latter finding may relate to the need for protection against Zn^{2+} inhibition in situations where anticoagulation is needed. The more than two orders of magnitude higher K_I values for the inhibition of factor Xa and thrombin by Zn^{2+} than for the inhibition of APC supports the conclusion that Zn^{2+} may regulate APC activity. In further support of this conclusion, it has been shown that APC inactivates factor Va much more efficiently on artificial phospholipid membranes or endothelial cells than on platelets (38). Cu^{2+} ions, which are also found in platelets, may contribute to such effect (39).

The inhibitory effect of Zn^{2+} on APC activity described here is in line with the overall procoagulant effect of Zn^{2+}, which has recently been reviewed (31). Deficiency of Zn^{2+} in the diet results in a decrease in the concentration of Zn^{2+} in blood and, as a consequence, leads to a tendency for clotting disturbances (40). For instance, the ion induces platelet aggregation and fibrin clot formation (40, 41). Moreover, Zn^{2+} has been proposed to lower the anticoagulant action of antithrombin, by enhancing the binding affinity of histidine-rich glycoprotein for heparin and thus neutralizing the anticoagulant activity of heparin in plasma (37). Apart from blood, protein C is expressed in several other tissues (http://www.proteinatlas.org). Another potential physiological role for Zn^{2+} inhibition of APC may thus be to prevent unwanted proteolytic activities in such tissues where the concentrations of zinc ions are high, including the reproductive system and various parts of the central nervous system, such as the hippocampus (32).

Interestingly, two of the proteins that APC interacts with have already been identified as Zn^{2+} binders. It was recently discovered that plasma protein S contains Zn^{2+} and that this ion is essential for the APC-independent anticoagulant activity of protein S (29). The finding that the Zn^{2+}-content in protein S preparations does not influence the APC-dependent

anticoagulant activity of protein S (29) is in line with our observation that the inhibitory

effect of Zn^{2+} on APC anticoagulant activity is caused by the ion binding to APC. Earlier it

was shown that α_2-macroglobulin is a Zn^{2+}-binding protein in blood (32). Additionally, other

serine proteases involved in the regulation of blood coagulation are affected by Zn^{2+},

including factor XII, factor XI and factor VIIa (31). For factor VIIa, the catalytic machinery

is inhibited by Zn^{2+} ions, as for APC. Two Zn^{2+} sites have been proposed in factor VIIa that

are distal from the catalytic site but overlap the Ca^{2+} binding site (42). The functional

consequence of the inhibitory effects of zinc ions on factor VIIa activity are, however,

opposite to those on APC activity, since factor VIIa functions as a procoagulant in the

presence of tissue factor. For APC, a procoagulant effect exerted by Zn^{2+} seems

straightforward to explain from a functional point of view, assuming that such inhibition

occurs at sites of injury where platelets accumulate and coagulation is required to prevent the

loss of blood. In contrast, the anticoagulant effects induced by the interaction of Zn^{2+} with

protein S and factor VIIa would make sense only if they occur at sites where coagulation is

not wanted, such as the healthy endothelium. Future investigations are required to clarify the

physiological functions of all these Zn^{2+}-protein interactions

The inhibitory effect of Zn^{2+} on APC activity may be contrasted with the activation of

the enzyme by Ca^{2+} (7, 8, 10), which raised the question of whether Zn^{2+} merely displaces

Ca^{2+} from its binding site, as for factor VIIa. This possibility could, however, be excluded for

several reasons. We have shown that the interaction of Zn^{2+} with APC is reversible, along

with the fluorescence change induced by the interaction of Zn^{2+} with APC. The decrease in

fluorescence induced by Ca^{2+}-binding to APC is only ~6% whereas the decrease in

fluorescence induced by Zn^{2+} binding is ~40%. Thus, if Zn^{2+} was binding to the Ca^{2+}-binding

site, an increase in fluorescence should be observed when excessive amounts of Ca^{2+} are

added to Zn^{2+}-bound APC. This does not happen. Moreover, the greater effect on the intrinsic

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fluorescence of APC induced by Zn^{2+} than by Ca^{2+} (8, 30) indicates that the two metal ions induce different conformational changes in APC. Finally, Zn^{2+} was able to completely abolish the amidolytic activity of APC, whereas Ca^{2+} somewhat stabilizes but is not required for the amidolytic activity of the enzyme (8). Since the K_I and K_D values observed for the interaction of Zn^{2+} with APC were 3-4-fold higher in the presence than in the absence of Ca^{2+}, it thus appears as if the Zn^{2+}-binding site is affected by the conformational change induced by Ca^{2+}. The similar influence of Zn^{2+} on des-Gla APC and full-length APC demonstrated that the Zn^{2+}-binding site is not located in the Gla domain, which contains several Ca^{2+}-binding sites. Together, these findings suggest that Zn^{2+} binds to a site that is distinct from the Ca^{2+} binding site in the 70-80 loop, but located, at least partially, in the serine protease domain.

To identify potential Zn^{2+}-binding sites, we sought appropriate residues (histidine, cysteine, aspartate and glutamate, accounting for 97% of amino acids in Zn^{2+} sites) (43) that are clustered in the structure of human APC or could be envisioned to be close after conformational changes. A careful consideration of PDB entry 1AUT (44) indicated several potential sites. The simplest possibility is illustrated in Fig. 6A & B. The structure of an engineered trypsin mutant with a 21 µM K_D for Cu^{2+} has been reported elsewhere (45), showing that the equivalent of His57 can assume a different conformation, allowing it to form part of an ion binding site. A similar motion of the charge-relay histidine was reported still earlier for Zn^{2+} binding in the tonin structure (46). In APC, possible zinc binding residues close to His57 include Asp60 and Glu60A. Since this histidine is an essential component of the catalytic triad, a change in conformation would render the enzyme inactive; this could easily occur without disrupting substrate binding, and so K_M. However, the large change in tryptophan fluorescence of APC on Zn^{2+} binding suggests that additional, more extensive conformational changes also occur. Looking for conserved residues in APC, we

found two clusters that are also candidates for ion-binding sites. One of these includes His48, Ser50, His107 and Trp51, as modeled in Fig. 6C. It is not clear whether the interaction of Zn^{2+} with these residues would affect the catalytic machinery. His91, His241 and Trp237 comprise another cluster, but since the latter residues are also present in other blood clotting proteases, they are not likely to give an effect specific for APC. These sites, however, include residues less commonly involved in Zn^{2+} binding. A potential Zn^{2+} site includes residues in the 144-153 loop, i.e. His144/Glu148/Glu149A, and possibly Asp18. The loop is an insertion in APC relative to other sequences, but the proposed Zn^{2+}-binding residues are not as strongly conserved in mammalian APC sequences as one would expect if the function is widespread. In this model, an extensive conformational change would be required to convey the information to the active site. Clearly, a number of structural, mutational and other studies will be required to understand the basis of zinc binding and inhibition.

The ability of Zn^{2+} to inhibit the amidolytic activity of APC is a highly interesting finding that broadens our understanding of the physiological function of this versatile serine protease, which is capable of inhibiting blood coagulation as well as inflammation and apoptosis. Furthermore, it is medically interesting from several points of view. APC is used clinically to treat patients with severe sepsis (47) and has the potential to be used in future treatments of several additional thrombotic and/or inflammatory diseases, including stroke (48), multiple sclerosis (49) and diabetic endothelial and glomerular injury (50). Knowledge about the Zn^{2+}-binding site may therefore be used to produce specialized recombinant forms of APC that are resistant to inhibition by Zn^{2+}. Such engineering could be combined with other tailor-made structural features of the enzyme to give a potentially improved therapeutic effect.

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Figure legends

Figure 1. **Effects of metal ions on the amidolytic activity of APC**. APC activity was

measured spectrophotometrically with the use of the chromogenic peptide substrate, S-2366,

as described in Materials and methods. The activity of APC measured in 100 μM EDTA in

the absence of metal ion was used as a control and was set to 100% activity. The values are

the means ± SE of two measurements.

Figure 2. **Amidolytic activity of APC and des-GLA APC measured**

spectrophotometrically with the substrate S-2366. (A) The influence of substrate

concentration on the rate of substrate hydrolysis by APC was determined in the presence of

100 μM EDTA (○), 10 μM Zn^{2+} (●), 2.5 mM Ca^{2+} (□) or 15 μM Zn^{2+} and 2.5 mM

Ca^{2+}(■). Solid lines represent nonlinear regression fitting to the Michaelis-Menten equation.

(B) The influence of the concentration of Zn^{2+} at 0.5 mM S-2366 on the amidolytic activity

of APC in the absence (○) and presence (□) of 2.5 mM Ca^{2+} and of des-Gla APC in the

absence of 2.5 mM Ca^{2+} (●) is shown. Solid lines represent nonlinear regression fitting to the

equation for non-competitive inhibition to give the inhibitor constant, K_I (Eq. 1) (17).

Figure 3. Influence of zinc ions on the inactivation of factor Va by APC

(A) Factor Va was incubated with APC in the presence of phospholipid vesicles and Ca^{2+} for

various time points in the absence (▲) or presence (△) of 100 μM Zn^{2+}, followed by the

determination of the remaining activity in a prothrombinase assay, as described in Materials

and methods. All values are the means ± SE of at least three measurements. The amount of

thrombin generated in the absence of APC was set to 100%. The solid lines represent

nonlinear regression fitting to a biphasic exponential function. (B) Factor Va was incubated

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with APC in the absence (∇) or presence ($\blacktriangledown$) of protein S. The assay, containing

phospholipid vesicles and Ca^{2+}, was run for 2 min at various Zn^{2+} concentrations, followed

by the determination of the remaining activity in a prothrombinase assay, as described in

Materials and methods. For each curve, the increase in the activity of thrombin generated is

expressed as % increase related to that generated in the absence of Zn^{2+}. The solid line for

data collected in the absence of protein S represents nonlinear regression fitting to a

hyperbolic binding equation. The values are the means $\pm$ SE of three measurements.

Figure 4. Zn^{2+}-**induced changes in tryptophan fluorescence and effects of EDTA**.

Uncorrected emission spectra determined at 25 °C as described in Materials and methods. (A)

The effect of 500 μM Zn^{2+} on the fluorescence of 200 nM APC and (B) 200 nM des-GLA

APC. (C) Effects of adding 200 μM EDTA to 200 nM APC in the absence or presence of 100

μM Zn^{2+}. Solid black lines, emission spectra of APC or des-Gla APC in the absence of Zn^{2+}

and EDTA; Dotted lines, emission spectra of Zn^{2+}-bound APC or des-Gla APC; Dashed

lines, emission spectra of APC containing EDTA; Solid gray lines, emission spectra of

EDTA added to Zn^{2+}-bound APC;

Figure 5. Equilibrium binding, studied by tryptophan fluorescence titrations, for the

interaction of Zn^{2+} with APC and des-Gla APC. Typical fluorescence titrations are shown

for the interactions of Zn^{2+} with APC in the absence ($\bigcirc$) and presence ($\square$) of 2.5 mM Ca^{2+}

and des-Gla APC in the absence of Ca^{2+} ($\bullet$). The concentrations used of APC and des-GLA

APC were 200 nM. Solid lines represent nonlinear regression fitting to the equilibrium

binding equation (15), assuming a 1:1 binding stoichiometry.

Figure 6. Models of Zn^{2+} binding to human APC.

(A) Stereo representations of human APC (PDB entry 1AUT) and anionic trypsin mutant

Arg96His (PDB entry 1AND) are shown in red and aquamarine, respectively. The Cu^{2+} ion

in trypsin is shown as a dark green sphere. (B) Model of the binding of Zn^{2+} (magenta

sphere) into the APC active site with only small conformational changes. (C) Model of Zn^{2+}

(yellow sphere) binding to a conserved APC site.

Table 1. Influence of Zn^{2+} on the inhibition constant, turnover number and Michaelis constant for the amidolytic activity of APC and affinity of APC for Zn^{2+}. The amidolytic activity of APC was measured spectrophotometrically with the use of the substrate S-2366, as described in Materials and methods. The data from determinations at different substrate concentrations in the presence of either 100 μM EDTA, 10 μM Zn^{2+}, 2.5 mM Ca^{2+} or 15 μM Zn^{2+} and 2.5 mM Ca^{2+} were fit to the Michaelis-Menten equation (Fig. 2A) to give the Michaelis constant, K_M, and turnover number, k_{cat}. The values ± SE were obtained by nonlinear regression fitting. Inhibitor constants, K_I, are the means ± SE of 3-4 plots of the amidolytic activity of APC and des-Gla APC versus [Zn^{2+}], determined in the absence or presence of 2.5 mM Ca^{2+} (Fig. 2B). Equilibrium dissociation constants, K_D, for the interaction of Zn^{2+} with APC are the means ± SE from 3-4 tryptophan fluorescence titrations (Fig. 5).

APC form	Additions	K_M (μM)	k_{cat} (s^{-1})	K_I (μM)	K_D (μM)
APC	EDTA	270±30	20±1	−	−
APC	Zn^{2+}	140±50	2.7±0.2	13±1	7.4±1.5
APC	Ca^{2+}	350±70	18±1	−	−
APC	$Ca^{2+} + Zn^{2+}$	360±80	10±1	57±4	20±3
Des-Gla APC	Zn^{2+}	nd[a]	nd[a]	25±3	19±3

[a] nd; not determined

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What is known about this topic?

- It is known that activated protein C (APC) has several binding sites for Ca^{2+}, one in the serine protease domain, one in the EGF-1 domain and nine in the GLA domain. Additionally, APC has one Na^+-binding site in the serine protease domain.
- It is known that binding of Ca^{2+} to the serine protease domain is required for the activation of protein C by the thrombin-thrombomodulin complex.
- The binding of Ca^{2+} and Na^+ to the serine protease domain allosterically regulate APC, resulting in enhanced enzymatic activity.

What does this paper add?

- This article shows that Zn^{2+} reversibly inhibits APC by a non-competitive mechanism. Inhibition occurs both with a chromogenic peptide and factor Va as substrates.
- This article further shows that the interaction of Zn^{2+} with APC is accompanied by a ~40 % decrease in tryptophan fluorescence. Apparent K_D and K_I values in the low micromolar range suggest that the inhibition may be relevant for the anticoagulant functions of APC.
- Finally, this article presents several amino acid clusters that are potential sites for Zn^{2+} binding.

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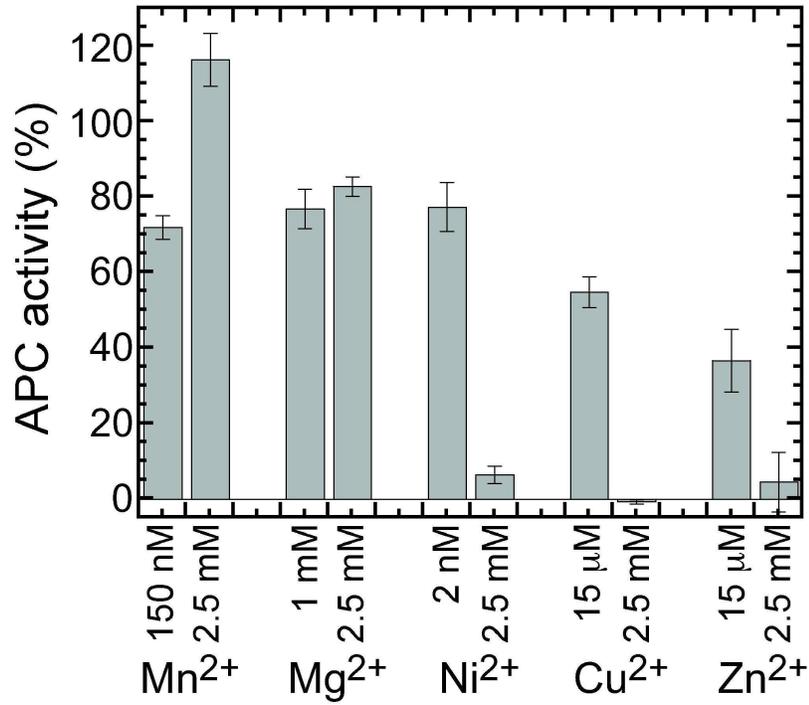
Fig.1. Zhu et al
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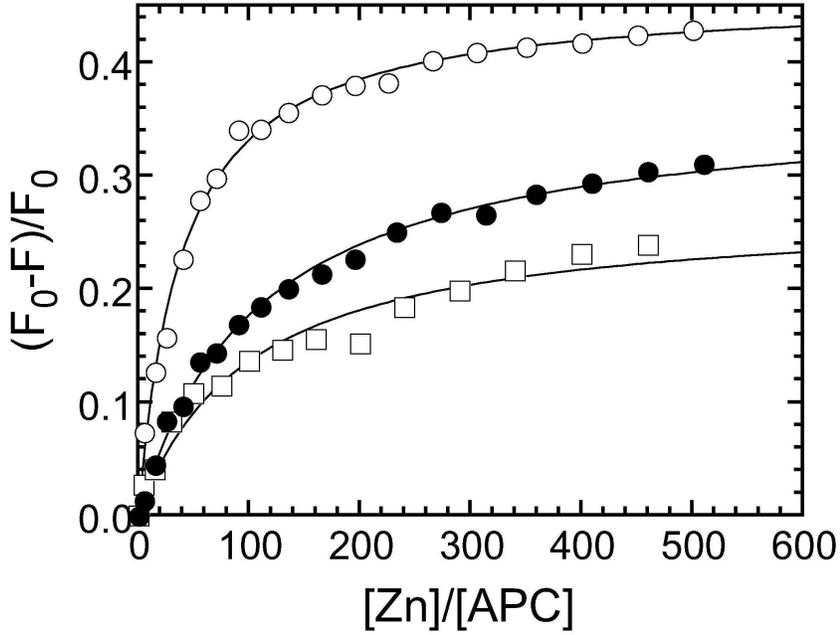
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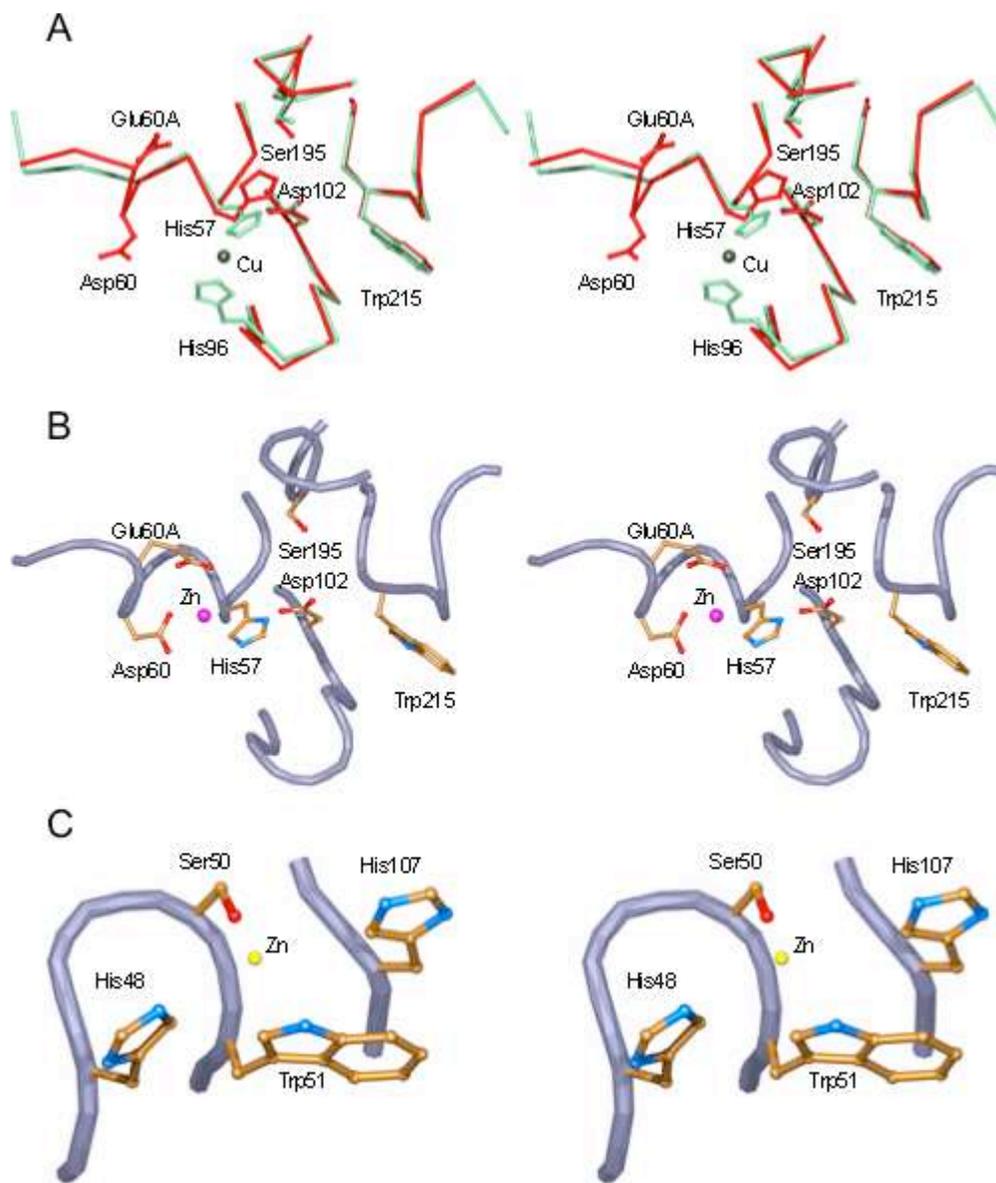
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