Blood Saving in Open Heart Surgery

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Editors

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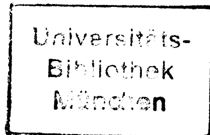

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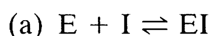
Aprotinin and its target enzymes in vitro and in vivo

H. Fritz, M. Jochum

1. Structure of aprotinin and kinetics of inhibition

The proteinase inhibitor aprotinin (synonym: basic pancreatic trypsin inhibitor, BPTI) is of a pear-shaped molecular form (Fig. 1). The inhibitor inactivates its target enzymes by formation of an enzyme-inhibitor complex. In this complex, the head region of the inhibitor ("reactive site", cf. Fig. 1) forms a tight contact with the cleft ("active site") of the proteinase, thereby blocking the proteolytic activity of the enzyme (1). As a structural pecularity of the aprotinin molecule, a lysine residue is located in position no. 15 of its amino acid chain (cf. Fig. 1). This lysine residue, which forms part of the reactive site of the inhibitor, fits perfectly into the corresponding specificity pocket of trypsin and of related proteinases, thus making aprotinin one of the most powerful inhibitors of trypsin-like proteinases.

The reaction between a proteolytic enzyme (E) and the corresponding inhibitor (1) is characterized by an equilibrium in which the formation rate of the complex (EI) from the single components is balanced by the dissociation rate of the complex into the parental molecules E and I (cf. equation (a))



(a) $E + I \rightleftharpoons EI$

The stability of the complex which reflects the affinity of the constituents for one another is given by the equilibrium constant K, specified by equation (b) where [E], [I] and [EI] are the molar concentrations of the enzyme, the inhibitor and the complex, respecitively:

(b) $$K_i = \frac{[E] \times [I]}{[EI]}$$

The dimension of the equilibrium constant K_i is mol/l [M]. The smaller the K_i value, the tighter the complex, i.e. minimum K_i values reflect maximum affinity. Typical K_i constants for complexes of aprotinin with various proteinases are compiled in Table 1. Among the various constants, the K_i value of 6×10^{-14} mol/l for the bovine trypsin-aprotinin complex is outstanding. The extremely

low K_i value indicates that the equilibrium of the reaction is shifted almost totally towards complex formation (cf. equation (a)) and therefore aprotinin is capable of completely inactivating bovine trypsin even at very low concentrations. The reason for this remarkable affinity is the perfect fit of the contact regions of trypsin and aprotinin which are complementary in shape ("key and lock" hypothesis). Not surprisingly, formation of the complex does not require engergy; instead, an appreciable amount of energy is released upon complex formation.

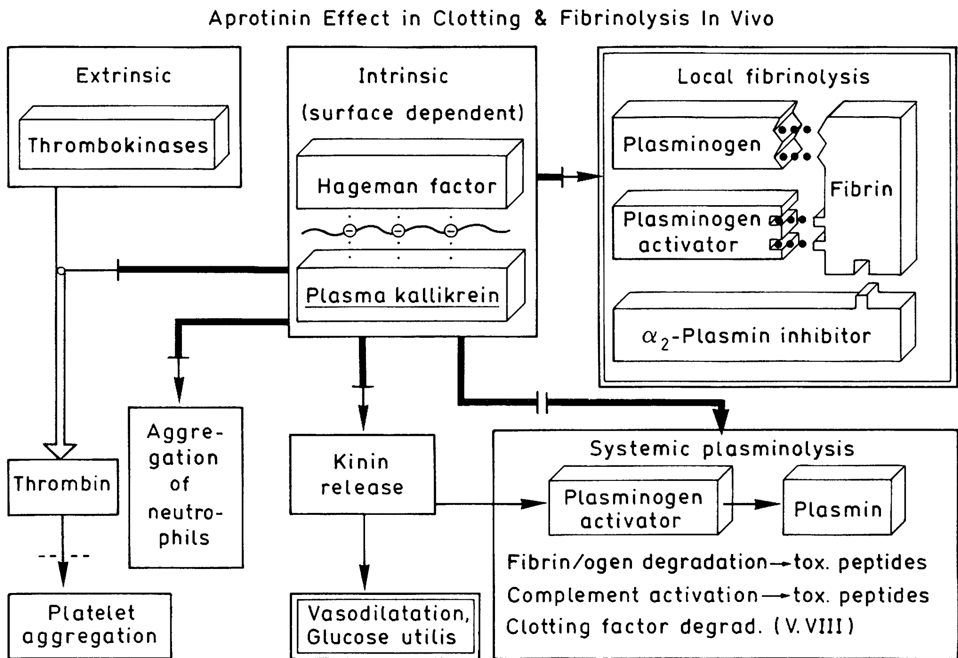


Fig. 1. The tertiary structure of aprotinin according to Huber et al. and Desienhofer & Steigemann (cited in Ref. 1).
The aprotinin molecule has a length of 29 Å, a diameter of 19 Å and contains a double-stranded antiparallel β-sheet structure (from Ala-16 to Gly-36) which is turned into a right-handed double helix with 14 amino acids per turn. Amino acids in the region of the hatched area (constituting the reactive site of the inhibitor) are in close contact with trypsin once the enzyme inhibitor complex is formed. Amino acid no. 19 (arginine) not contained in the contact area plays a major role in the complex formation with the kallikreins due to its inherent basic character (cf. Ref. 1).
Positively charged amino acid side chains are present at positions 1, 15, 17, 20, 39, 39, 41, 42, 46, 53 ($n = 10$); negatively charged ones at positions 3 and 50 only.

Table 1. Equilibrium constants K_i of enzyme-inhibitor complexes. For full references, cf. Ref. 1.

enzyme	source	species	K_i (mol/l)	pH
trypsin	pancreas	cow	6.0×10^{-14}	8.0
anhydrotrypsin	pancreas (a)	cow (a)	3.0×10^{-13}	8.0
trypsinogen	pancreas	cow	1.8×10^{-6}	8.0
chymotrypsin	pancreas	pig man	9.0×10^{-9} 6.0×10^{-9}	8.0 7.2
plasmin	plasma	pig man man	4.0×10^{-9} (b) 2.3×10^{-10} (b) 1.0×10^{-9}	8.0 7.8 7.3
kallikrein	pancreas pancreas submand. gland urine plasma urine plasma	pig pig pig pig pig man man	1.0×10^{-9} (b) 1.3×10^{-9} (b) 1.6×10^{-9} (b) 1.7×10^{-9} (b) 1.0×10^{-7} 0.9×10^{-10} 3.0×10^{-4}	8.0 9.0 9.0 9.0 7.8 8.0 8.0
elastase	leukocytes	man	3.5×10^{-6} (c)	8.0
urokinase	urine	man	8.0×10^{-6} (d)	8.8

(a) semisynthetic enzyme
(b) approx. values only
(c) strongly dependent on the ion concentration (cf. Ref. 1)
(d) Dietl T, Hugo B, Fritz H, unpublished results

2. Effective aprotinin levels in vivo

Aiming at clinical applications of aprotinin or related inhibitors, it is of **prime importance to know which aprotinin concentration (s) can be reached with a given dosage regimen.** For a long time, precise assays to quantitate aprotinin accurately in patients' plasma have been unavailable. To overcome this problem, we have developed two assays which allow the measurement of aprotinin concentrations in plasma and related biological fluids with high specificity and accuracy. Both assays, i.e. an enzymatical test (2) and an enzyme immunoassay (3), are suitable for routine measurements. Meanwhile, the tests have been successfully applied to monitor aprotinin plasma concentrations in several clinical trials.

In a first clinical study carried out in polytraumatized patients (4) **a continuous infusion of 250.000 KIU/h (kallikrein inhibitor units per hour)** equivalent to approx. 35 mg aprotinin per hour **resulted in a mean plasma concentration of 50 KIU/ml (almost equalling 1 μmol/l).** This value can be appreciated if compared with the plasma levels of endogenous proteinase inhibitors (Table 2). The concentrations of the major plasma proteinase inhibitors vary from approx. 1 μmol/l (α_2-plasmin inhibitor, α_2PI) to approx. 52 μmol/l (α_1-proteinase inhibitor, α_1PI, previously known as α_1-antitrypsin). Hence, an aprotinin concentration of 1 μmol/l inhibitor is of the same order of magnitude as that of the endogenous α_2PI.

The level of α_2PI in human plasma is low compared to that of other naturally occurring inhibitors (cf. Table 2). However, the α_2PI concentration can be elevated by inflammatory stimuli with a resulting 1.6-fold increase of the α_2PI plasma level. This increase in plasma concentration is brought about by the enhancement of α_2PI biosynthesis in the liver which is usually a matter of a few days. On the other hand, a major part of the endogenous α_2PI pool can be rapidly "consumed" during sepsis, polytrauma, disseminated intravascular coagulation (DIC), or reactive fibrinolysis. Under these conditions, restoration of the anti-plasmin pool by administration of an exogenous inhibitor such as

Table 2. Proteinase inhibitors of human plasma, their relative molecular masses (M_r) and their mean plasma concentrations (acute phase proteins are in italics).

Abbreviation	inhibitor	Mr × 1000	mean concentration mg/100 ml	mol/l
α_2M	α_2-macroglobulin	725	260	3.6
α_1PI	α_1-*proteinase inhibitor*	50	260	52
α_1AC	α_1-*antichymotrypsin*	70	45	6.4
ITI	inter-α-trypsin inhibitor	160	45	2.8
αPI	α-cysteine-proteinase inhibitor	60	50	8
β_1CI	β_1-collagenase inhibitor	40	1.5	0.4
AT III	antithrombin III	65	26	4.0
α_2PI	α_1-*plasmin inhibitor*	70	6	0.9
CI INA	*CI-inhibitor* (CI-inactivator)	100	24	2.4

aprotinin seems useful with a resultant plasma concentration equalling that of the natural inhibitor under physiologcial or pathophysiological conditions (0.5–1.6 μmol/l). Thus, the rationale of our approach is to establish a sufficiently high inhibitor level under "consuming" conditions, thereby compensating the loss of the endogenous inhibitor potential to prevent an overshoot of (potentially harmful) proteolysis right from the start. Left to the body itself, the "filling-up" of the inhibitor potential would take at least some days.

3. Target enzymes of aprotinin in vitro and in vivo

The question arises as to which enzymes are effectively inhibited by an aprotinin plasma concentration of 1 μmol/l. This consideration is important to evaluate the potential therapeutic efficacy of such an aprotinin medication.

A. Plasmin

The K_i for the complex of human plasmin and aprotinin is very low (approx. 2.3×10^{-10}mol/l). In order to assess the efficacy of aprotinin in inhibiting plasmin in vivo, the actual concentrations of the enzyme and the inhibitor have to be considered. It is reasonable to assume that only a minor fraction of the plasminogen present in the plasma (concentration approx. 2 μmol/l) is activated even under pathological conditions. Therefore, at a given aprotinin concentration of 1 μmol/l, one would expect the inhibitor concentration to clearly exceed the actual plasmin concentration, thus allowing a rapid and (almost) complete inhibition of plasmin liberated systemically into circulation. Also, one should keep in mind that a certain fraction of systemically released plasmin is inhibited by the endogenous inhibitors.

Proper inhibition of systemically released plasmin (i.e. the plasmin fraction *not* locally effective on the fibrin fibre) is mandatory because this enzyme effectively digests plasma factors ("plasminolysis"). Among other target proteins, the systemically released plasmin can split and thereby inactivate important plasma factors (e.g. coagulation factors V and VIII, fibrinogen, fibronectin), or activate them (e.g. the complement factors C1, C3 and C5). Especially under the conditions of DIC, systemic plasminolysis contributes to the proteolytic breakdown of essential clotting factors, and, as a consequence, loads the reticulo-endothelial system (RES) with protein degradation products.

Principally, we have to consider two pathways of plasmin action: first, the pathological plasminolyis e.g. of fibrinogen ("fibrin*ogen*olysis"), and second, the physiologically important fibrinolysis, a process targeted at and restricted to fibrin. In the latter case, natural activators of plasminogen are concentrated on fibrin fibres of the clot, thus allowing local activation of plasminogen and subsequent degradation of fibrin in situ. From a clinical point of view, inhibition of local fibrinolysis by aprotinin is normally unwanted and an aprotinin plasma concentration of 1 μmol/l is unlikely to prevent local fibrinolysis effectively for two reasons: (i) the plasmin concentration in situ (i.e. on the clot) is considerably higher than the systemic plasmin concentration, and (ii) the access of fibrin-associated plasmin by aprotinin is sterically restricted (5). Experimental studies to assess these hypotheses are still under way.

B. Tissue kallikrein

Human tissue kallikreins have affinities to aprotinin comparable to those of plasmin (K_i approx. 1×10^{-10}mol/l). Since the tissue kallikreins are present in plasma in much lower concentrations (approx. 3×10^{-10}mol/l) than plasmin, complete blockage of their enzymatic activities at 1 μmol/l aprotinin is highly likely. However, rigorous experimental proof of this hypothesis awaits further investigations.

C. Plasma kallikrein

Unlike the tissue kallikreins, human plasma kallikrein is much less efficiently inhibited by aprotinin, the K_i value of the complex being 3.0×10^{-8} mol/l. This equals the amount of plasma kallikrein that can be generated from its precursor under pathological conditions (e.g. approx. 1.5×10^{-8} mol/l if 10 % of the total plasma kallikrein is activated). It is still an open question as to what extent the proteolytic activity of the plasma kallikrein against its natural substrates, i.e. high-molecular-weight kininogen and Hageman factor (F XII), can be inhibited by 1 μmol/l aprotinin. Theoretical considerations predict that only aprotinin concentrations as high as **4 μmol/l (200 KIU/ml) will properly inhibit liberated plasma kallikrein** (6).

In this context, the notion of Schapira et al. (7) that plasma kallikrein can stimulate the aggregation of polymorphonuclear (PMN) granulocytes is worth mentioning. Aggregation of the PMN granulocytes may result in a massive shedding of lysosomal proteinases such as elastase, and according to Schapira et al. this effect is directly induced by plasma kallikrein and *not* mediated via

kinins. Typically , the lysosomal proteinases generate significant amounts of proteolytic breakdown products due to "unspecific" proteolysis of plasma proteins. This process is entailed by a rapid loading of the RES which functions in the elimination of these products. Present evidence suggests that this cascade of events triggered by plasma kallikrein has more harmful than beneficial effects on homeostasis.

Assuming that Schapira's notion (7) can be confirmed by others, a triad of functions has to be envisaged for plasma kallikrein:
first, it liberates kinins from kininogens;
second, it triggers the endogenous pathway of blood coagulation via F XII activation;
and third, it stimulates PMN granulocytes and induces release of lysosomal enzymes. Hence, **proper regulation of plasma kallikrein activity by inhibitors under pathological conditions would be of special significance.**

D. Trypsin

The K_i value for the complex of bovine trypsin and aprotinin (approx. 10^{-14} mol/l) is extremely low. By analogy, one might conclude that human trypsin (both the cationic and anionic form) is equally well inhibited by aprotinin, though the exact K_i for this complex remains to be determined. Keeping in mind that the pancreas is the only source for trypsin, it is reasonable to assume that the plasma concentration of trypsin remains well below 1 μmol/l even under pathological conditions. Hence, 1 μmol/l aprotinin should suffice to completely inactivate trypsin accidentally released from the pancreas under inflammatory conditions, e.g. in acute pancreatitis.

E. Various enzymes

The affinity of aprotinin for lysosomal proteinases from polymorphonuclear (PMN) leukocytes such as elastase or cathepsin G is too low (cf. Table 1) to ensure proper inhibition of these enzymes even if they are present in very small amounts. When these enzymes are liberated from the granulocytes by the stimuli of a severe trauma or a massive infection, the body supplies a number of far more efficient inhibitors, e.g. α_1 PI and α_2M for elastase and α_1AC, α_2M α_2 and α_1 PI for cathepsin G (cf. Table 3). Similar characteristics apply to urokinase

Tab. 3. Target enzymes of natural plasma proteinase inhibitors (for acronyms. cf. Table 2).

Primary target enzymes: lysosomal and pancreatic proteinases	
α_2M	all classes of proteinases, in particular – pancreatic trypsin – elastase and cathepsin G from neutrophils – plasma kallikrein and plasmin – cysteine proteinases and collagenases
α_1PI	serine proteinases – elastase from neutrophils – pancreatic chymotrypsin and elastase – pancreatic trypsin
α_1AC	serine proteinases – cathepsin G from neutrophils – mast cell chymase
ITI	serine proteinases – pancreatic trypsin and chymotrypsin
αCPI (a)	cysteine proteinases – cathepsin H, L, B, S – calpain
β_1CI	metalloproteinases – collagenase
Primary target enzymes: cascade proteinase	
AT III	– thrombonin and factor Xa
α_2PI	– plasmin (cf. α_2M)
CI INA	– plasma kallikrein (cf. α_2M) – Hageman factor – Clr. Cls

(a) The two cysteine proteinase inhibitors of human plasma (α_1-CPI and α_2-CPI) are identical with HMW and LMW kininogens (Müller-Esterl et al): Human plasma kininogens are identical with α-cysteine proteinase inhibitors. Evidence from immunological, enzymatical and sequence data. FEBS Letters 182: 310–314, 1985).

and chymotrypsin (cf. Tables 1, 3 and Ref. 1). There are, however, derivatives of aprotinin available which strongly inhibit lysosomal elastase (8); their therapeutic efficacy remains to be examined.

4. Aprotinin effects unrelated to inhibitor function

Apart from its role as an enzyme inhibitor, aprotinin possesses another extraordinary feature, i.e. an unusually high positive net charge at pH 7.4 (the

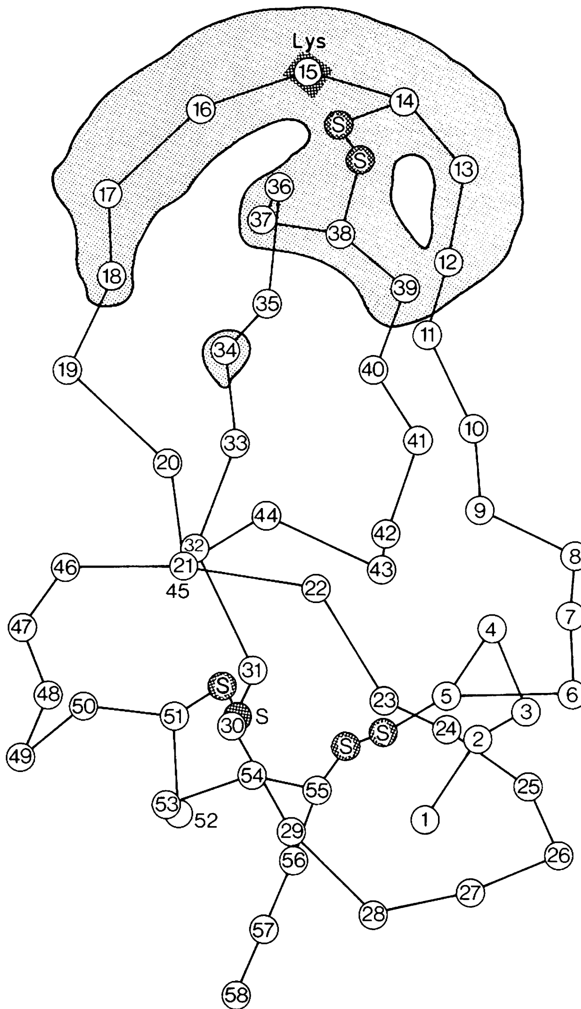


Fig. 2. In vivo effects of aprotinin in the blood coagulation cascade and the fibrinolytic system.
Interference of aprotinin with the endogenous coagulation pathway can be observed at high plasma concentrations of the inhibitor. Simultaneously, the aggregation of neutrophils, the liberation of kinins, and the kallikrein-mediated activation of plasminogen are slowed down. At very high aprotinin plasma concentrations, even platelet aggregation is reversibly inhibited. Unlike the endogenous pathway, the extrinsic blood coagulation cascade is virtually unaffected by aprotinin.
Systemic fibrinolysis (cf. text for details) is effectively inhibited even at moderate concentrations of aprotinin. Also, the plasmin-mediated degradation of many plasma factors and hence the formation of harmful degradation products is largely prevented. By contrast, local fibrinolysis is not significantly affected at moderate aprotinin concentrations, and only blocked at very high concentrations of the inhibitor. Unlike the endogenous plasmin inhibitor α_2PI which is trapped into the evolving fibrin network, the access of aprotinin to the formed clot seems to be restricted.

isoelectric point of aprotinin is 10.5). No negatively charged amino acid residues are present at the apical region of the pear-shaped molecule (cf. Fig. 1) which thus forms a typical dipole. Mediated by ionic interactions the inhibitor protein binds to negatively charged substances or surfaces. This effect is classified "unspecific" as opposed to the inhibition of proteinases which is a "specific" feature of the molecule. The rapid and almost selective uptake of aprotinin by phagocytes of kidney tissue is thought to be mediated by this kind of ionic interaction. Also, storage of aprotinin in its natural host cells, i.e. the bovine mast cells, is believed to be mediated by ionic binding of aprotinin to negatively charged heparin molecules abundant in these cells.

One of the effects related to the dipole character of aprotinin is the inhibition of platelet aggregation which is observed at very high aprotinin concentrations (above 8 μmol/l equivalent to approx. 400 KIU/ml). Here, aprotinin most probably binds to negatively charged surface molecules of the platelet membrane (9). Similarly, ionic interactions with aprotinin can lead to the stabilization of a fluid cell membrane which might aid in maintaining certain cell functions for a limited time span. This effect is exploited for the long-term storage of blood where aprotinin is added as a stabilizer. Characteristically, the effects of aprotinin based on ionic interactions are fully reversible once the concentration of the protein falls below a certain level. – For possible future applications it is important to keep in mind that the preserving effect of aprotinin on global cell functions has been convicingly demonstrated only for platelets (9). Moreover, only exceptionally high aprotinin concentrations rarely obtained in vivo for short periods of time will promote the observed effects.

5. Summary

The aim here was to show that the molecular properties of aprotinin and the mechanisms by which it interacts with other molecules are known in considerable detail. Figure 2 provides an illustrative sketch of our ideas on the basic mechanisms of aprotinin action and their potential application under clinical conditions. With the advent of specific assay systems, the quantification of aprotinin in biological fluids of diverse origin as well as tissue extracts has become possible. This achievement allows the precise determination of aprotinin levels reached in vivo by well defined dosage regimens, and the correlation of these levels to desirable or attainable therapeutic effects of the inhibitor.

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The biochemical features and potential applications of aprotinin and its target enzymes have been extensively reviewed elsewhere (1); the reader ist referred to the references therein.

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