THE PHYSIOLOGICAL INHIBITORS OF BLOOD COAGULATION AND FIBRINOLYSIS

D. Collen, B. Wiman and M. Verstraete Editors

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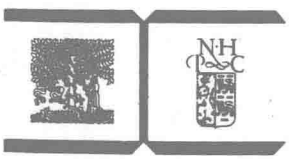
Proceedings of a Round-Table Conference held at the University of Leuven, Belgium, July 22-23, 1978

Editors
D. Collen et al
B. Wiman
and
M. Verstraete



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PREFACE

This book contains the proceedings of a Round-Table Conference on the Physiological Inhibitors of Coagulation and Fibrinolysis, which was held at the University of Leuven, Belgium on July 22 and 23, 1978. This meeting consisted of a group of 26 investigators who have been actively engaged in research in this field and was organized in order to exchange, discuss and critically evaluate recent developments in this field. During the last few years significant and rapid progress has indeed been made in our knowledge of the identity, physicochemical properties, reaction mechanism and physiological role of the plasma proteins which neutralize enzymes of the coagulation or fibrinolytic systems.

To make this symposium useful to the participants as well as to the scientific community, rapid publication of the proceedings of this meeting has been undertaken. It is hoped that the new ideas and hypotheses put forward in these proceedings will stimulate further productive research in this field.

We are grateful to the participants in this meeting for their cooperation, for their willingness to provide manuscripts of their presentations and for their intense discussions which in our opinion have made this meeting a success.

This symposium has been made possible by a grant from the Nationaal Fonds voor Wetenschappelijk Onderzoek.

D.C.
B.W.
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Leuven, Belgium, Sept. 1, 1978

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The Physiological Inhibitors of Coagulation and Fibrinolysis,
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INTRODUCTION TO THE ROUND-TABLE CONFERENCE

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Human plasma contains several proteins which inactivate proteolytic enzymes. The main properties of the best characterized protease inhibitors are summarized in Table I.

TABLE I
Survey of the protease inhibitors from human plasma.

Name	Concentration (mg/100 ml)	Mr	Amino acid content (percent)	Carbohydrate content (percent)	Chain composition (number)
α_1-antitrypsin	290 ± 45	54,000	86	12	1
α_1-antichymotrypsin	49 ± 7	69,000	73	25	1
inter-α-trypsin inhibitor	50	160,000	90	8	
antithrombin III	24 ± 2	65,000	85	13	1
C_1^--inactivator	24 ± 3	104,000	65	35	1
α_2-macroglobulin	260 ± 70	725,000	92	8	4
α_2-antiplasmin	7 ± 1	70,000	87	13	1
inhibitor of plasminogen activation		80,000			

A number of reviews on these protease inhibitors have recently appeared (1-3). These inhibitors, with the exception of α_2-macroglobulin, form 1:1 stochiometric enzymatically inactive complexes with proteases. The exact mechanism of the inhibitor-protease reaction is not yet fully proven, but evidence is accumulating that the mechanism of interactions may be of similar nature to that eludicated between proteases and low molecular weight inhibitors of plant or animal origin : namely, formation of a covalent bond between a reactive center residue of the inhibitor (usually a basic amino acid) and the active center serine of the protease.

The protease inhibitors react at very different rates with the different proteases and can on this basis be classified as rapid and slow inhibitors. Antithrombin III is a progressive inhibitor of several proteases but its reaction rate is markedly increased in the presence of heparin. α_2-antiplasmin is a rapid inhibitor of plasmin but a slow inhibitor of several other plasma proteases.

The action mechanism of α_2-macroglobulin differs from that of the other plasma protease inhibitors. Protease-α_2-macroglobulin complexes are very poorly reactive with high molecular weight substrates but still active against low molecular weight substrates, which suggests that large substrate molecules are excluded by steric hindrance.

Several of the plasma protease inhibitors listed in Table I can inhibit proteolytic enzymes of the coagulation and fibrinolytic systems. At present it is believed that antithrombin III and α_2-macroglobulin are the primary physiological inhibitors of thrombin and the recently described fast-acting plasmin inhibitor (cfr. this volume, chapter by S. Müllertz) and α_2-macroglobulin are of importance for the neutralization of plasmin formed in plasma.

In agreement with the participants, this meeting will deal with our present knowledge, ideas, hypotheses and speculations on the physicochemical properties, reaction mechanisms and physiological roles of the plasma proteins which neutralize selected enzymes of the coagulation and fibrinolytic systems. The inhibitors which will be discussed are antithrombin III, the fast-acting plasmin inhibitor and α_2-macroglobulin and in addition the less well defined inhibitor(s) of plasminogen activation. In view of the different nomenclature used by several authors and for the sake of uniformity it was agreed to use the following abbreviations throughout the text of all manuscripts (but not in the titles) : AT for antithrombin or antithrombin III, AP for α_2-antiplasmin, antiplasmin, α_2-plasmin inhibitor or primary plasmin inhibitor and α_2M for α_2-macroglobulin.

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ACTIVATION, ACTION AND INHIBITION OF TRYPSINOGEN-TRYPSIN

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Protein proteinase inhibitors are ubiquitous, they have been found in all kinds of animals and plants as well as in bacterials. Especially in the case of serine proteinases a huge variety of inhibitors with quite different sequences and molecular weights has been characterized and assigned to several families of homologous inhibitors, as e.g. the Kunitz PTI family, the secretory Kazal Family, the Bowman-Birk Legume Family and the Kunitz STI Family (1). In spite of their different shapes and magnitudes almost all of these inhibitors seem to obey the same standard mechanism that has been most lucidly studied and established for the interaction of STI (2, 3) and PTI (4,5,6,7,8) with porcine and bovine trypsin respectively. Only some of the inhibitors from mammalian sera, being particularly discussed at this conference, appear to be exceptions from this common inhibition type. Also, the mechanism of activation of trypsinogen, upon which we have recently obtained some quite surprising and interesting structural results (9,10), may be common to all serine proteinases including the more complicated proteinases of coagulation and fibrinolysis. Amino acid sequence studies of some of these proteinases show the presence of a C-terminal trypsin-like core with large segments attached to it which modify the specificity and are responsible for secondary interactions with other macromolecules (11). Because trypsin is in so many ways a prototype, a detailed inderstanding of its structure and function, activation and inhibition may be of more general relevance.

Trypsin and Trypsinogen Structure

Fig. 1 shows the spatial arrangement of the single chain of bovine β-trypsin (12,13) represented by its α-carbons. Its structure is very similar to that of α-chymotrypsin (14) with its N-terminal and C-terminal halves mostly organized in two adjacent barrels. In the cleft between, most of the reactive residues are arranged, i.e. the Ser 195 - His 57 - Asp 102 triade, forming the charge-relay-system. Close to the reactive Ser 195 opens the specificity pocket with the Asp 189 at its bottom. On the opposite side of the molecule is a single bound Ca^{2+}-ion within an external loop ("Calcium-loop") (12). This Ca^{2+} is quite important for the trypsin stability. About 25 internal bound

water molecules are integral constituents of the protein structure (12), most of them being also found in trypsinogen (9,10) and in chymotrypsin (14).

The cationic bovine trypsinogen contains additional six amino acid residues at its N-terminus (Val 10 - Asp 11 - Asp 12 - Asp 13 - Asp 14 - Lys 15)(15,16). Only after the cleaving off of this N-terminal hexapeptide the newly liberated Ile 16 - Val 17 - Gly 18 - N-terminus can insert into the molecule to form the internal salt bridge with the Asp 194 carboxylate group. 85 % of the structures of trypsin and its proenzyme are identical. The corresponding amino acid residues are linked by double lines in Fig. 1. Most remarkably, the charge-relay residues have almost identical conformations in both free molecules being in neither case typical for the active state due to interaction with the surrounding solvent molecules (10).

Only four segments (indicated by single lines in Fig. 1) with the N-terminus to Gly 19, Gly 142 to Pro 152, Gly 184 to Gly 193 and Gly 216 to Asn 223, are in a completely different state in the zymogen : there is no significant electron density for these segments in the Fourier map of trypsinogen as demonstrated for Gly 140 - Trp 141 - Gly 142 in Fig. 2. Flexibility starts rather abruptly in single residues. In five of the seven hinges where chains become flexible a glycine residue is located. These glycines are conserved in serine proteases, suggesting similar structural transitions upon activation. Three of the seven hinges in trypsinogen have fixed aromatic residues adjacent to glycine. These four chain segments which are flexible in trypsinogen form a tightly interdigitating structural unit after activation peptide cleavage in active trypsin. We call it therefore "activation domain" (9). The triggering event seems to be the conformational change of Asp 194 which forms a link to His 40 in trypsinogen but an internal salt bridge with the newly formed N-terminal Ile 16 in trypsin (Fig. 3a,b).

The conformational linkage between the Ile 16 cleft and the specificity pocket can be demonstrated by inducing the structural transition from both sides. PTI e.g., due to its high binding affinity to the trypsin state, rigidifies upon complexation both the specificity pocket and the Ile 16 cleft as observed crystallographically in the trypsinogen-PTI-complex, although there is no Ile 16 N-terminus (17).

According to CD evidence (18,19), this rigidification also occurs in trypsinogen upon the concerted binding of the covalently bound p-guanidinobenzoate group in the pocket and of free Ile-Val in the Ile 16 cleft. As shown in Fig.4

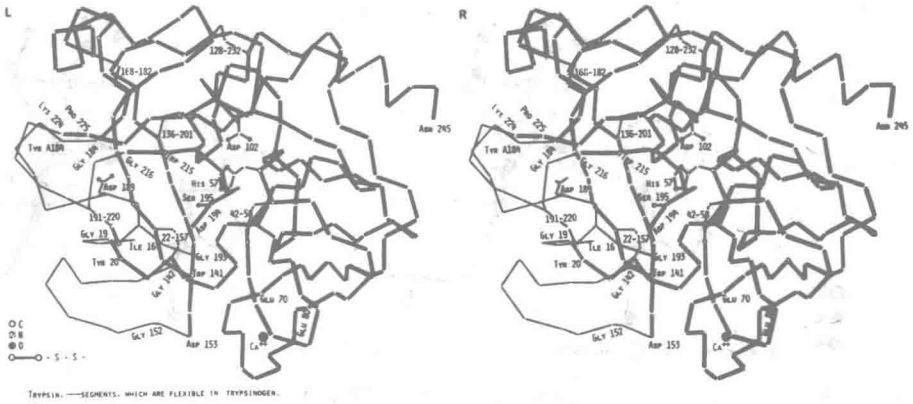

Fig. 1. Stereo drawing of the C^{α} carbon positions in trypsin. Residues linked by single lines are flexible in trypsinogen, those linked by double lines are fixed (10).

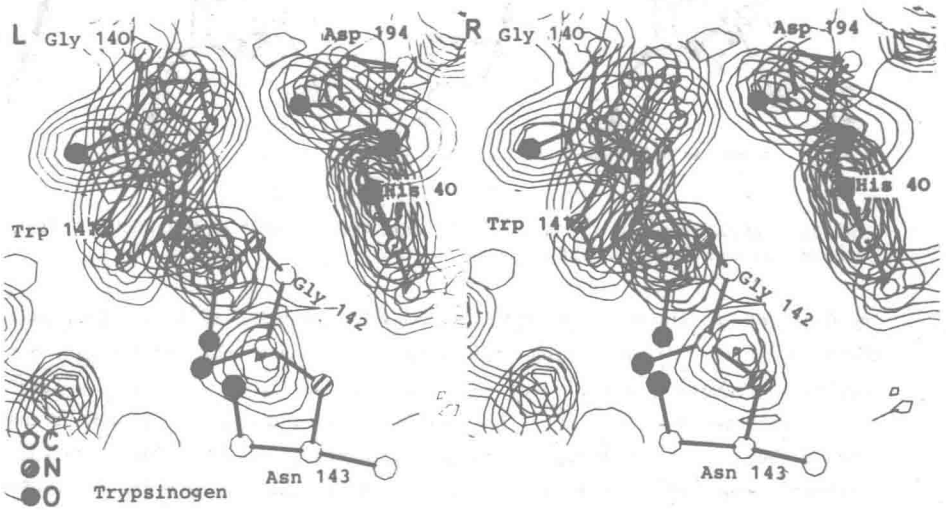

Fig. 2. Stereo pair of a section of the final Fourier map of trypsinogen with the refined trypsinogen model around Gly 140 - Trp 141 - Gly 142 overlaid (17).

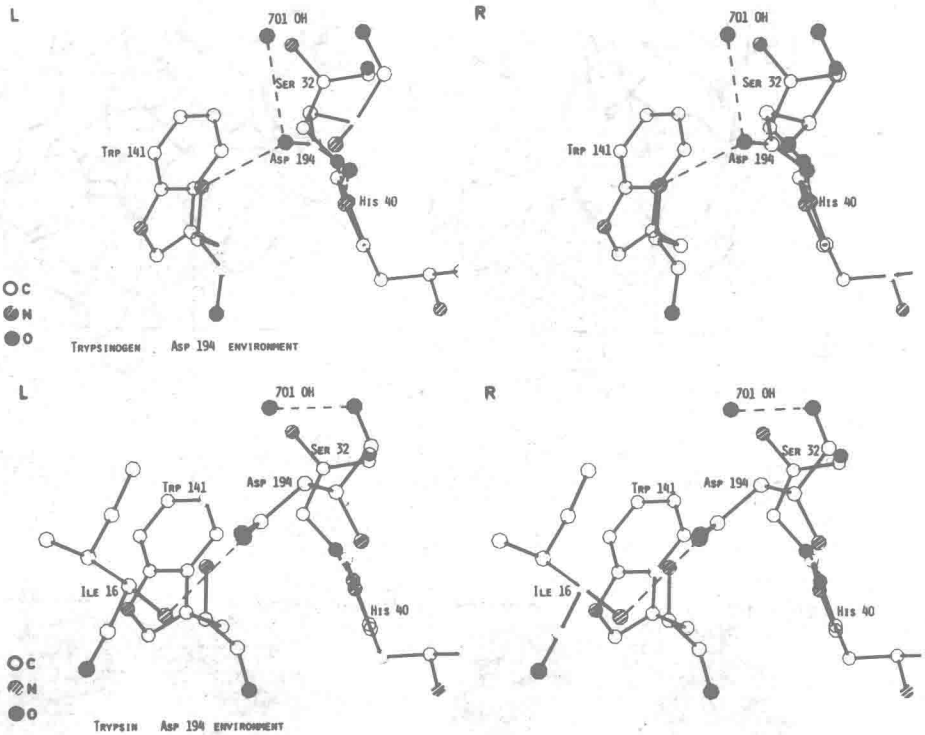

Fig. 3. Stereo drawing of the Asp 194 conformation and environment in trypsinogen (a) and trypsin (b) (10).

the CD-signal of the bound pGB-group becomes identical to that of pGB-trypsin above 10^{-4} M Ile-Val indicating a complete transition of pGB-trypsinogen to the trypsin conformation upon binding of Ile-Val in the Ile 16 cleft. Sequentially related dipeptides as Ile-Leu, Val-Val, Ile-Ala etc. are also efficient transformers, but only at much higher peptide concentrations. Dipeptides with the N-terminal sequences of various trypsinogen species as e.g. Ala-Pro or Phe-Pro, are completely inefficient in inducing this conformational transition. Thus the activation peptides of trypsinogen serve as masking elements besides their role as a specific cleavage site for enterokinase (20).

Consequently, according to our crystallographic and spectroscopic evidences, the single chain molecule trypsinogen shows up to be a typical allosteric protein. Fig. 5 shows the different states for the trypsinogen-PTI-Ile-Val-system and for the trypsin-PTI-system respectively, together with experimentally

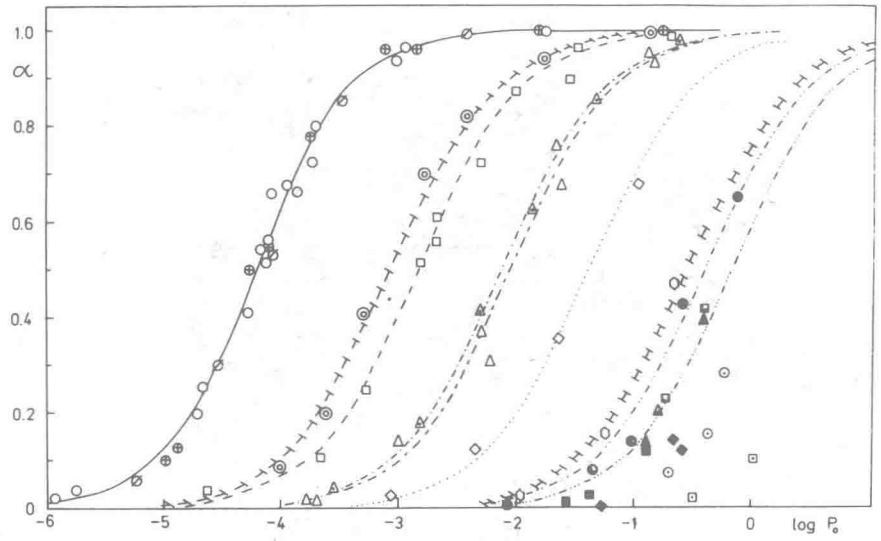

Fig. 4. Transformation of pGB-trypsinogen (1.9×10^{-5} M) into a trypsin-like conformation upon peptide binding according to circular dichroisms (19).
(—○—) Ile-Val ; (—⊕—) Ile-Val-Gly-NH_2 ; (—∅—) Ile-Val-Gly-O-Met ; (—◎—) Ile-Leu ; (- -□- -) Val-Val ; (—▲—·) Ile-Ala ; (— -△- —) Leu-Val.

determined (large numbers) or estimated (small numbers) equilibrium and binding constants. Trypsinogen/trypsin can principally exist only in two different, interconvertible structures. The species observed crystallographically are A, B, C, D and E. The equilibrium between the trypsinogen and trypsin conformations can be estimated to be about 10^8 in favour of the partially ordered zymogen structure. Due to the very high affinity for the Ile 16 cleft this large positive free energy of 9 kcal/mol can be more than compensated by binding of the "own" Ile 16 - Val 17-N-terminus. As can be seen the induction ability of the "foreign" Ile Val is far inferior. Allosteric induction of actity without activation peptide cleavage may be of physiological significance in some trypsinogen related zymogens. So, e.g., plasminogen appears to develop proteolytic activity upon complex formation with streptokinase prior to activation. Likewise the complement component C1r seems to attain some activity upon binding to the immunoglobulin-C1q-complex. Even an inhibitory mechanism based on the opposite transformation of the active serine proteases into their presumably partially ordered conformers would be conceivable. This happens at higher pH-values due to deprotonation of the Ile 16 - ammonium group.

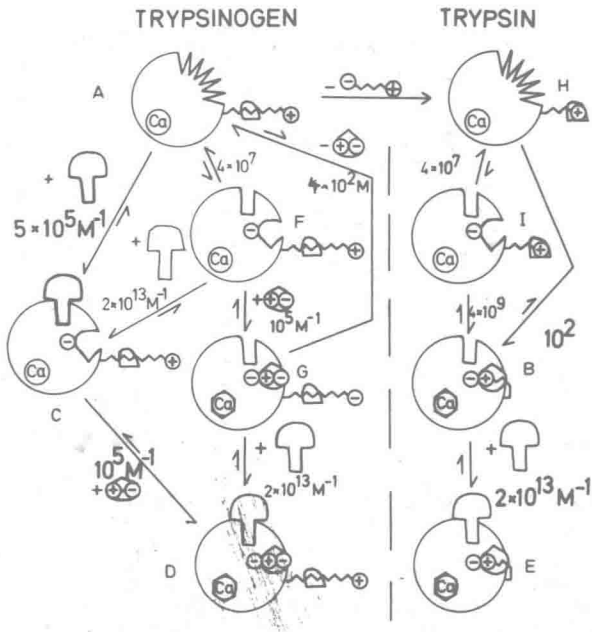

Fig. 5. Equilibrium scheme for the trypsinogen/PTI/Ile Val-system (left) and the trypsin/PTI-system (right) at pH 8.0, 0.02 M Ca, 20°C. (∿∿) flexible segments, ( ) PTI, ( ) Ile-Val(19).

Trypsin-Trypsin Inhibitor Complex

The basic pancreatic trypsin inhibitor (PTI) is a small, pear-shaped protein molecule (21) of the Kunitz type. It is an extremely potent inhibitor of trypsin and other proteases, binding trypsin with an association constant above 10^{13} M^{-1} (22). It has recently been shown that as in the case of other natural inhibitors (1), interaction of PTI with proteases involves a limited proteolytic cleavage at the active site bond leading to an equilibrium state between virgin and modified PTI (23). Thus inhibitor-protease interaction involves catalytic action. PTI and other natural inhibitors have the characteristic of excellent substrates in the association step. They inhibit by the virtue of their slow dissociation.

In the trypsin-PTI-complex only a small part of both molecules is in contact: 14 amino acid residues out of 58 of the inhibitor, and 24 residues out of 223 of trypsin. The contact is characterized by a complicated network of hydrogen bonds, by more than 200 van der Waals contacts and by 8 intermolecular water molecules. The binding area is tightly packed with a density identical to that