INHIBITION OF BLOOD COAGULATION FACTORS BY SERINE ESTERASE INHIBITORS

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Received 14 June 1972

1. Introduction

The reaction mechanism of blood coagulation presumably comprises a series of proenzyme–enzyme conversions. In a strict sense this has only been proven for prothrombin (factor II) which has been shown upon activation to yield the serine esterase thrombin [1].

The coagulation factors II, VII, IX and X have much in common, both in the way of synthesis (dependence upon vitamin K) and physicochemical properties [2]. It therefore is conceivable that these four factors are proenzymes of serine esterases, that is they are serine esterases when in their activated forms*.

It has been shown that factor X_a can split organic esters and is inhibited by DFP [3]. No enzymatic properties have been found to the factors V_a and $VIII_a$ [18, 19]. We set out to investigate the inhibitory action of a set of 25 known serine esterase inhibitors on the activities of coagulation factors II, V, VII, VIII, IX and X with the purpose of finding out which of these were serine esterases. We also hoped to find specific inhibitors for each factor which would greatly facilitate further research in blood coagulation.

* The active form will be indicated by the subscript a, so factor II_a = thrombin etc.

2. Materials and methods

2.1. *Serine esterase inhibitors*

The serine esterase inhibitors were chosen from those described by Myers and coworkers [4, 5]. As the material had been stored for 15 years, their ability to inhibit rat brain aliesterase was tested (according to [6]) and compared to the original activity as given by Myers et al. [4, 5]. Of the 78 inhibitors 52 had kept their original activity. 2-NO_2-4-carboxyphenyl-*N*-*N*-diphenylcarbamate (NCDC) was prepared according to Erlanger [8] in the laboratory for organic chemistry (Head, Prof. Dr. E. Havinga) in Leiden.

2.2. *Coagulation factor assays and preparations*

Activities of factors II, VII, V and X and thrombin were determined in one-stage assays as described in [13]. Factor VIII and IX activities were estimated as described by Veltkamp et al. [9], and factor X_a was determined by mixing 0.1 ml sample, 0.1 ml Seitz filtered oxalated bovine plasma (deficient in factors VII and X) and 0.1 ml phospholipid suspension. After 30 sec 0.1 ml 25 mM $CaCl_2$ was added and the clotting time was measured. Factor VII_a was tested like factor VII, but with the use of bovine brain thromboplastin instead of human material. Due to the species specificity of the factor VII-activating enzyme in the thromboplastin, this test only measures human material that has been activated, i.e. VII_a. Factor VII_a, X_a and IX_a preparations were obtained by purification from human serum according to the procedures described by

Table 1

No.	Structure	Molar concentration	Inhibition (%) of factor II_a	VII_a	X_a
1	*p*Cl-Ph-O-C(= O)-NH-Me	2×10^{-4}	17	0	20
2	Ph-O-C(= O)-NH-Me	2×10^{-4}	0	0	20
		5×10^{-4}	0	0	25
3	Ph-O-C(= O)-NH-Et	5×10^{-4}	5	0	15
		10^{-3}	30	20	20
4	Ph-O-C(= O)-NH-Pr	5×10^{-4}	10	0	10
		10^{-3}	20	10	20
5	*p*Cl-Ph-O-C(= O)-NH-Ph	10^{-4}	20	10	< 5
		2×10^{-4}	30	15	< 5
6	*p*Me-Ph-O-C(= O)-NH-Me	10^{-4}	20	5	< 5
		2×10^{-4}	30	5	< 5
7	$o NO_2$-Ph-O-C(= O)-N-Me_2	4×10^{-4}	0	0	0
8	$o Me_3$-N-Ph-O-C(= O)-NH-Me	2×10^{-4}	0	0	0
9	$m NH_2$-Ph-O-C(= O)-$N(CH_3)_2$	2×10^{-3}	35	0	0
10	$m NO_2$-Ph-O-C(= O)-$N(CH_3)_2$	4×10^{-4}	25	0	10
11	$m(Me_2$-$CH(CH_3))$. Ph-O-C(= O) $NHCH_3$	2×10^{-4}	5	0	0
12	Ph_2-N-C(= O)-O-Ph-$o NO_2 p$COOH	2×10^{-4}	45	30	0
		4×10^{-4}	70	33	0
		10^{-3}	80	35	0
13	$(p NO_2$-Ph-$O)_2$-P(= O)-Ph	10^{-5}	> 95	30	0
		2×10^{-5}	> 95	55	15
14	$(p NO_2$-Ph-$O)_2$-P(= O)-Cyc. hex.	5×10^{-5}	65	0	0
		10^{-4}	65	0	0
		1.5×10^{-4}	90	0	5
15	$(p NO_2$-Ph-$O)_2$-P(= O)-Isoprop.	10^{-5}	80	< 5	< 10
		2×10^{-5}	> 95	10	< 10
		4×10^{-5}	> 95	20	< 10
16	$(p NO_2$-Ph-$O)_2$-P(= O)-eth.	10^{-5}	> 95	25	0
		2×10^{-5}	> 95	30	0
17	$(p NO_2$-Ph-$O)_2$-P(= O)-CH_2-Phe	2×10^{-5}	> 95	> 65	40
		5×10^{-5}	> 95	> 65	50
18	$(p NO_2$-Ph-$O)_2$-P(= O)-O-Ph	4×10^{-5}	> 95	30	5
		10^{-4}	> 95	40	< 10
19	$(p NO_2$-Ph-$O)_2$-P(= O)-NH-Me	10^{-5}	50	0	0
		2×10^{-5}	60	0	0
20	$(Cl$-Ph-$O)_2$-P(= O)-NH_2	2×10^{-4}	> 95	0	0
		4×10^{-4}	> 95	0	< 10
		10^{-3}	> 95	40	10
21	$(Cl$-Ph-$O)_2$-P(= O)-NH-Me	2×10^{-4}	15	0	0
		5×10^{-4}	20	0	0
22	$(Cl$-Ph-$O)_2$-P(= O)-NMe_2	5×10^{-4}	70	20	20
		10^{-3}	85	40	25
23	$(p NO_2$-Ph-$O)_2$-P(= O)-NH-Phe	2×10^{-4}	35	0	0
		5×10^{-4}	40	0	0
24	$(p NO_2$-Ph-$O)_2$-P(= O)-NMe_2	10^{-4}	15	< 5	< 5
		2×10^{-4}	10	< 5	< 5
25	$(Ph$-$O)_2$-P(= O)-NH-Me	2×10^{-4}	0	0	0
		5×10^{-4}	10	0	0

Table 2

No.	Structure	Molar concentration	Inhibition (%) of factor II_a	V	VII_a	VIII	IX_a	X_a
21	$(Cl\text{-}Ph\text{-}O)_2\text{-}P(=O)\text{-}NHCH_3$	10^{-4}	0	0	0	0	3	0
19	$(pNO_2\text{-}Ph\text{-}O\text{-})_2\text{-}P(=O)\text{-}NH\text{-}CH_3$	2×10^{-5}	60	0	0	0	10	0
11	$m(Me_2\text{-}CH(CH_3))$. Ph-O-C(= O)$NHCH_3$	2×10^{-4}	5	0	20	0	0	0
26	$CH_3\text{-}Ph\text{-}O\text{-}C(=O)\text{-}NHCH_3$	2×10^{-4}	0	0	0	0	20	20
16	$(pNO_2\text{-}Ph\text{-}O)_2\text{-}P(=O)$-eth	2×10^{-5}	95	0	40	0	0	5
1	p-Cl-Ph-O-C(= O)-NH-Me	2×10^{-4}	20	0	0	0	0	20
2	Ph-O-C(= O)-NH-Me	10^{-3}	0	0	30	0	0	25
17	$(pNO_2\text{-}Ph\text{-}O)_2\text{-}P(=O)\text{-}CH_2\text{-}Ph$	4×10^{-5}	95	0	65	0	< 5	50
27	$CH_3\text{-}Ph\text{-}O\text{-}C(=O)\text{-}NH\text{-}Ph$	4×10^{-4}	30	0	45	0	< 5	0

Swart [11]. A factor V preparation was obtained according to Kahn [19], factor VIII was prepared after Van Mourik et al. [12].

The starting material for the preparation of thrombin was a concentrate of the factors II, VII, IX and X from plasma. This preparation was activated by addition of human brain thromboplastin (1% v/v) and dialysis against 12.5 mM $CaCl_2$ in 0.01 M Tris-HCl buffer pH 7.0 at 4° for 16 hr. This material was applied to a DEAE-Sephadex column in 0.01 M Tris-HCl buffer pH 7.0, and eluted stepwise with increasing concentrations of NaCl in the buffer. The fractions eluted between 0.05 M and 0.10 M contained a 90% pure thrombin. The reference curve for the coagulation factor assays was obtained in dilutions of normal plasma at an acetone concentration of 0.5% (v/v).

2.3. *Assessment of the inhibition*

The inhibitors were dissolved in acetone. In all experiments and controls pure acetone was added so as to obtain a final concentration of 0.5% (v/v). The experiments were carried out as follows: At 37° a sample of the coagulation factor preparation was incubated with the inhibitor at the desired concentration. A blank was done in parallel. The decrease in the blank was taken to represent nonspecific inactivation (that is, denaturation of the coagulation factor). Coagulation factor determinations were carried out after 3 hr of incubation.

In order to avoid misinterpretation of the results by an effect of the inhibitor on the coagulation factor assay, the coagulation time was determined immediately after addition of the inhibitor and after 3 hr of incubation. No, or only a marginal, inhibition was observed at zero time.

3. Experimental results

In table 1 are given the results obtained with a series of 25 carbamates and phosphoric esters on coagulation factors II_a, VII_a and X_a. None of these inhibited coagulation factors $V_{(a)}$ and $VIII_{(a)}$. Not all of these factors were tested on factor IX_a. Table 2 summarizes the effect of a selected group of inhibitors on the factors II_a, V, VII_a, VIII, IX_a, and X_a.

4. Discussion

The fact that the four coagulation factors II, VII, IX and X are inhibited by a series of compounds known to act on the active serine residue in esterases and proteolytic enzymes strongly argues in favour of their being serine esterases. The fact that these factors are inhibited to a markedly different extent by different substances proves that they are individual moieties. As factors V and VIII are not inhibited, they probably are not serine esterases.

The coagulation factor esterases have a remarkedly narrow specificity. Factors II_a and X_a can split tosyl-arginine methyl ester and benzoyl arginine methyl ester [3, 14] but the natural substrates of these enzymes are other coagulation factors. Factor II_a (thrombin) acts on fibrinogen and factor XIII. Factor VII_a has only one proven action: conversion of th zymogen factor X in its active form (i.e. factor X_a).

The same holds true for factor IX_a. Factor X_a in its turn can only act upon factor II to convert it into thrombin.

It has been shown that the factors VII_a, IX_a and X_a as such cannot act on their natural substrates. Factors IX_a and X_a need to be adsorbed onto a phospholipid surface (via a Ca-ion). Next to the factor acting as an esterase, another factor has to be adsorbed: factor V together with X_a; factor VIII with factor IX_a [14–17]. The factors V and VIII could not be shown to have enzymatic properties [18, 19]. With factor VII the situation is less clear, it has been proven, however, that for its action a protein intimately bound to a lipid structure is necessary, both of which are derived from wounded cells [20].

It thus appears that the moieties active in blood coagulation consist of a serine esterase, adsorbed on a surface next to a protein (tentatively called paraenzyme [21]) that in itself is inactive, but which determines the specificity of the esterase.

For the mode of action of the paraenzyme two possibilities can be put forward:

a) it causes changes in the tertiary structure of the esterase, so as to alter its specificity
b) it provides accessory binding sites for the substrate so as to ensure matching of the vulnerable site on the substrate to the active site of the esterase.

This problem is at present under investigation.

References

[1] E. Shaw, M. Mares-Guia and W. Cohen, Biochemistry 4 (1965) 2219.
[2] M.I. Barnhart and G.F. Anderson, Federation Proc. 20 (1961) 52.
[3] M.P. Esnouf and W.J. Williams, Biochem. J. 84 (1962) 62
[4] D.K. Myers, Thesis, Amsterdam (1954).
[5] D.K. Myers, A. Kemp, J.W. Tol and M.H.T. de Jonge, Biochem J. 65 (2) (1957) 232.
[6] W.W. Umbreit, P.H. Burris, J.F. Stauffer, in: Manometric Techniques and Related Methods for the Study of Tissue Metabolism (Burgess Publ. Co., Minniapolis, Minn., USA).
[7] D.K. Myers, Thesis (1954) p. 7.
[8] B.F. Erlanger and F. Edel, Biochemistry 3 (1964) 346.
[9] J.J. Veltkamp, E.A. Loeliger and E.F. Drion, Thromb. Diath. Haemorrhag. 18 (1968) 403.
[10] M.J.P. Kahn and H.C. Hemker, Thromb. Diath. Haemorrhag. Vol. XXVII, No. 1 (1972) 25.
[11] A.C.W. Swart, B.H.M. Klaasen, C.H.F. Bloys van Treslong-De Groot and H.C. Hemker, Thromb. Diath. Haemorrhag., accepted for publication.
[12] J.A. van Mourik and I.A. Mochtar, Biochim. Biophys. Acta 221 (1970) 677.
[13] H.C. Hemker, E.A. Loeliger and J.J. Veltkamp eds., Human Blood Coagulation (Leiden University Press, 1969).
[14] H.C. Hemker, M.P. Esnouf, P.W. Hemker, A.C.W. Swart and R.G. Macfarlane, Nature 215 (1967) 248.
[15] H.C. Hemker and M.J.P. Kahn, Nature 215 (1967) 1201.
[16] P.G. Barton, C.M. Jackson and D.J. Hanahan, Nature 214 (1967) 923.
[17] C. Hougie, K.W.E. Denson and R. Biggs, Thromb. Diath. Haemorrhag. 18 (1967) 211.
[18] S. van Creveld, I.A. Mochtar and C.N. Pascha, Thromb. Diath. Haemorrhag. 17 (1967) 188.
[19] M.J.P. Kahn and H.C. Hemker, Coagulation Vol. III, No. 1 (1970) 63.
[20] Y. Nemerson, Biochemistry 5 (1966) 601.
[21] H.C. Hemker and M.J.P. Kahn, Abstracts Fifth Meeting FEBS, Prague, 1968, no. 350.