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Observations concerning the substrate specificity of Arvin

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SUMMARY

Arvin, a thrombin-like serine proteinase, was found to have a methyl esterase specificity limited to esters of L-arginine and its N^{α}-substituted acyl derivatives. Binding of other L-amino acid esters by Arvin and bovine thrombin was detected by studying their competitive effects on the conversion of fibrinogen to fibrin. Binding of L-lysine methyl ester by Arvin decreased with N^{α}-acyl substitution, whereas with thrombin, more clotting inhibition was observed. The observed esterase characteristics of Arvin were used in determining its inability to activate human plasminogen.

Arvin★, a thrombin-like enzyme obtained from *Agkistrodon rhodostoma* venom, is of clinical interest as a potential anticoagulant agent[1]. It functions physiologically by destroying plasma fibrinogen and inducing a safe, artificial afibrinogenemia without apparent effects on other blood coagulation factors[2].

Arvin was isolated by the method of Esnouf and Tunnah[3] followed by chromatography on Amberlite IRC-50[4]. Its esterolytic activity with reference to 20 readily available L-amino acid methyl esters (Cyclo Chemical) was studied by the method of Siegelman *et al.*[5]. Samples were removed periodically from incubation systems containing 0.02 M substrate and 1–30 units★★/ml of Arvin in 0.15 M NaCl, buffered at pH 8.0 with 0.05 M $Na_2B_4O_7$ and maintained at 37°C. These samples as well as the corresponding controls from mixtures incubated without Arvin were assayed for methanol and the control values subtracted in each case to obtain actual hydrolysis rates. The methyl esters of L-proline, L-alanine, L-tryptophan, L-tyrosine and the S-containing amino acids gave

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★★One Arvin unit approximately equivalent to one N.I.H. thrombin unit.

intense colors by this method. Better results were obtained when the methanol from a sample of each incubate was transferred at very low pressure into a U-tube cooled in liquid nitrogen.

Fibrinogen clotting time inhibition was determined by briefly preincubating 0.1 ml of 0.20 M L-amino acid methyl ester with 0.1 ml of Arvin or Thrombin Topical, Parke-Davis (5 units/ml) and then adding 0.8 ml of a saline solution of bovine fibrinogen (Pentex, 65% clottable) to give a final fibrinogen concentration of 0.2% (w/v).

The esterase activity data are shown in Table I. Significant substrate hydrolysis was observed only with L-arginine methyl ester. Under the conditions described, Arvin-catalyzed breakdown of other substrates was less than 5% of that observed with L-arginine methyl ester.

TABLE I

L-AMINO ACID ESTERASE ACTIVITY OF ARVIN
All incubations were carried out at 37°C in 0.05 M sodium tetraborate, pH 8.0.

Substrate	*Final Arvin concn (units/ml) (A)*	*Incubation time (min) (t)*	*Relative esterase activity★ x 10^{-3}*
L-Arginine-OMe	5	10	2.8
α-Acetyl-L-arginine-OMe	1	7	13.7
L-Lysine-OMe	10	78	0.031
α-Acetyl-L-lysine-OMe	10	78	0.027
L-Tyrosine-OMe	30	120	0.072
α-Benzoyl-L-histidine-OMe	10	100	0.018
L-Cystine-di-OMe	30	120	0.040
L-Phenylalanine-OMe	10	100	0.015
L-Glutamic acid-di-OMe	10	100	0.040
Methyl esters of L-alanine, L-hydroxyproline, L-valine, α-tosyl-L-lysine, L-serine, L-proline, L-isoleucine, L-threonine, L-leucine, glycine, L-methionine, L-cysteine, L-tryptophan, L-histidine, α-tosyl-L-ornithine	10	100	<0.015

★Difference in absorbance (580 nm) of incubate and blank divided by $(A) \times (t)$.

Larger amounts of Arvin essential for estimation of other potential substrate specificities, such as reported for thrombin[6] were not available. However, a comparison with bovine thrombin was made of the extent of binding of various L-amino acid esters by the coagulant enzymes in both preparations. This was done by using fibrinogen clotting times as an index of free enzyme (Arvin or thrombin) concentration in preincubates of enzyme and various L-amino acid esters. The data recorded in Table II represent the

TABLE II

EFFECTS OF L-AMINO ACID ESTERS ON ARVIN AND THROMBIN CLOTTING ACTIVITIES

L-Amino acid ester	*Concn x 10^2 (M)*	*Arvin*	*Thrombin*
L-Aspartic-OMe	2.4	0	0
Glycine-OMe	2.5	0	0
L-Serine-OMe	2.5	0	0
L-Valine-OMe	2.5	0	0.38
L-Isoleucine-OMe	2.5	0	0.39
L-Hydroxyproline-OMe	2.5	0.06	0.09
L-Threonine-OMe	2.5	0.03	0.33
L-Leucine-OMe	2.5	0.05	0.27
L-Glutamic-di-OMe	2.5	0.06	0.32
L-Proline-OMe	2.5	0.21	0.18
L-Alanine-OMe	2.5	0.32	1.20
L-Cysteine-OMe	2.4	0.23	0.57
L-Cystine-di-OMe	2.0	0.25	1.03
L-Methionine-OEt	2.5	0.15	0.72
L-Tyrosine-OMe	2.5	0.27	1.50
L-Phenylalanine-OMe	2.5	0.11	1.67
L-Tryptophan-OMe	2.5	0.14	2.15
L-Histidine-OMe	2.0	0.45	0.37
L-Lysine-OMe	2.5	0.57	0.86
L-Arginine-OMe	0.5	1.83	1.85
	2.5	9.15★	9.25★
α-Tosyl-L-arginine-OMe	0.04	0.52	1.50
	2.5	32.50★	94.00★
α-Tosyl-L-ornithine-OMe	2.5	0	3.90
α-Tosyl-L-lysine-OMe	2.5	0.15	8.20
ϵ-Tosyl-L-lysine-OMe	2.5	0.41	4.00

★Estimated values based on respective dilution factors.

proportional increases in clotting times resulting from addition of ester to the system, *i.e.* clotting time after preincubation with ester *minus* clotting time in the absence of ester divided by the latter (blank) clotting time. Plots of clotting time against inhibitor concentration were linear, in agreement with the assumption of just one type of binding site (Fig. 1). The method is specific for only the actual clotting enzyme activities, there being no interference from other enzymes.

From the data in Table II it is apparent that of the unsubstituted L-amino acid esters tested L-arginine methyl ester is the best inhibitor of both Arvin and thrombin clotting activities. Thrombin clotting activity was also inhibited by some L-amino acids esters other than those of L-arginine and L-lysine. Although inhibition by aromatic L-amino acid esters was not unexpected in view of the hydrolysis of these substrates by higher concentrations of thrombin[3], it is of interest that N^{α}-substituted aromatic L-amino acid nitrophenyl esters were hydrolyzed more rapidly by Arvin than by thrombin[3]. In view of Arvin's lower affinity for, or decreased inhibition by, aromatic L-amino acid methyl esters,

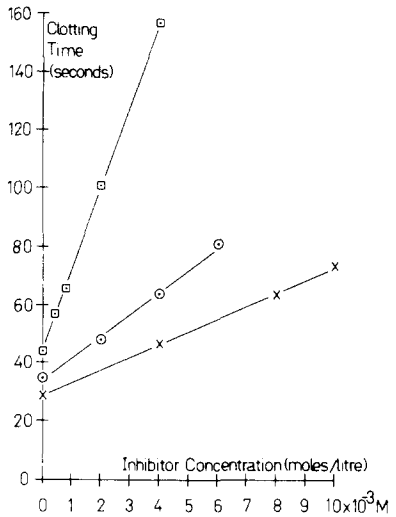

Fig. 1. Clotting time plotted against inhibitor concentration for 0.2% fibrinogen solutions at 37°C and pH 7.4 containing the following L-amino acid esters as inhibitors: ⊡, N^{α}-tosyl-L-arginine methyl ester; ⊙, L-arginine methyl ester; ×, N^{α}-acetyl-L-arginine methyl ester.

this effect indicates a higher k_3 in Arvin-catalyzed substrate breakdown than in that promoted by thrombin. This interpretation is supported by the finding that deacylation of *p*-guanidinobenzoate-Arvin is far more rapid than that of the corresponding thrombin derivative (T. Exner and J.L. Koppel, unpublished observations).

For both Arvin and thrombin, clotting inhibition by L-lysine methyl ester was considerably smaller than that due to L-arginine methyl ester. This relatively low affinity may explain why Sherry *et al.*[7], in their initial study, did not observe the L-lysine ester specificity of thrombin. Tosyl N^{α}-substitution increased inhibition of thrombin clotting activity by L-lysine methyl ester, but had the opposite effect on Arvin's clotting activity. With L-arginine methyl ester, on the other hand, tosyl N^{α}-substitution increased binding with both enzymes – an effect confirmed by iso-pH titration data (T. Exner and J.L. Koppel, unpublished observations). There is also the possibility that L-arginine methyl ester and, in fact, some of the apparently non-hydrolyzable substrates, may function in some other way to inhibit clotting, *e.g.* by preventing polymerization of fibrin monomers.

Except for the effects produced by L-proline methyl ester and L-histidine methyl ester, Arvin's clotting activity was generally inhibited less than that of thrombin. This may be due to a higher affinity of Arvin for binding sites on fibrinogen or to its lower affinity for esters of L-amino acids generally. Some of the latter substrates, *e.g.* L-histidine methyl ester, which were not hydrolysed appreciably by Arvin and only slowly hydrolysed by thrombin[6], were found to noticeably increase clotting times, thus indicating, presumably, their binding by enzyme. Clearly, in the absence of measurable binding by enzyme, catalytic activity cannot be determined, regardless of how large the individual rate constants may be subsequent to the initial formation of the *ES* complex. However, as soon

as there is measurable binding, and no matter how small k_3, this may still be thought of as involving "specificity" for that particular L-amino acid ester. Under physiological conditions, the natural abundance of protruding amino acid side chains would tend to preclude enzymatic attack on groups with weaker affinities.

Arvin's limited specificity was found to be useful in determining whether or not it can activate plasminogen. Plasmin, like thrombin, rapidly hydrolyses tosyl-L-lysine methyl ester[8], whereas Arvin, even in high concentrations, is without effect. Thus the generation in incubates of plasminogen and Arvin of a tosyl-L-lysine methyl esterase activity can only be due to the formation of plasmin. Arvin, at a final concentration of 20 units/ml, and human plasminogen[9] at a final concentration of 20 casein units/ml, were incubated in 0.15 M NaCl, 0.05 M Tris–HCl, pH 7.5, at 37°C for up to 2 h. Samples were removed periodically and tested for tosyl-L-lysine methyl esterase activity by pH-stat titration. None was in fact found. Streptokinase activation of plasminogen occurred rapidly under the same conditions and was readily detected by this procedure.

Thus, the thrombolytic effects noted by Reid and Chan[1] and the fibrin breakdown products observed by Bell *et al.*[2] following Arvin therapy may result from plasmin activity generated by a different mechanism. This may conceivably involve Arvin activation of plasma pre-kallikrein and subsequent activation of plasminogen by kallikrein, analogous to the indirect Hageman Factor effect on the fibrinolytic mechanism[10].

The very limited specificity shown by Arvin is of interest, both theoretically and practically. Its apparently restricted physiological effects may be due to its much lower affinity for aromatic amino acid side chains considered as possible sites for the secondary effects exhibited by thrombin[11]. From the theoretical point of view, comparison with other trypsin-like enzymes, such as thrombin, plasmin and kallikrein, points up the remarkable specificity of Arvin for L-arginine esters. The reason for this selectivity may lie in some variation of the amino acid sequence near the active centre, known to be similar in all these enzymes, or it may be related to other steric factors not yet appreciated.

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