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INSENSITIVITY OF FACTOR V AND FACTOR Va TO DIISOPROPYLFLUOROPHOSPHATE AND ANTITHROMBIN III

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Summary

Bovine plasma Factor V and Factor Va, the latter prepared by thrombin or venom activator action on Factor V, are not inactivated by diisopropylfluorophosphate or antithrombin III nor do they form identifiable complexes with either of these reagents. On the basis of these data, it is concluded that bovine Factor V and Factor Va are not serine proteases.

The intrinsic system of blood coagulation in mammals can be represented in general as a scheme in which a coagulant protein exists in the normal state in a zymogen form and is converted to an active enzymatic form during coagulation. Some of these coagulant proteins have been identified as serine proteases [1]. Until recently two proteins, Factor V and its "activated" form, Factor Va, and Factor VIII and its "activated" form, Factor VIIIa, were considered to be high molecular weight co-factors and not to be zymogens or to possess enzymatic activity (at least serine protease activity). Davie, E.W. (personal communication) noted that in his laboratory Factor VIIIa was sensitive to diisopropylfluorophosphate. This observation prompted initiation of experiments in this laboratory designed to determine if Factor V or Factor Va interacted with diisopropylfluorophosphate and also with antithrombin III. During the course of our studies, Vehar and Davie published their observations in which they concluded Factor VIIIa was a serine protease [2]. The results of our study show that Factor V and Factor Va are not sensitive to diisopropylfluorophosphate or antithrombin III and at least by these criteria cannot be classified as serine proteases.

Crude bovine thrombin was purchased from Parke-Davis and purified to near homogeneity according to the procedure of Lundblad [3]. Russell's vi-

Abbreviations: Factor Va^T, Factor V activated by thrombin; Factor Va^V, Factor V activated by a protease from Russell's viper venom; PPO, 2,5-diphenyloxazole.

per venom was purchased from the Miami Serpentarium. A single preparation of human plasma antithrombin III (the generous gift of Dr. Earl W. Davie) was used in all antithrombin III experiments. Sephadex G-200 and CL Sepharose 6-B were obtained from Pharmacia Fine Chemicals. Acrylamide and bis-acrylamide of electrophoresis quality were products of BioRad. Coomassie Brilliant Blue R, heparin (sodium salt from porcine intestinal mucosa) and sodium diethyl barbiturate were products of Sigma Chemical Co. Other reagents were purchased from Fisher Chemical Company.

[1-^{3}H]Diisopropylfluorophosphate (spec. act. 0.9 Ci/mmol, 0.21 mg/ml) and Protosol were purchased from New England Nuclear. All experiments involving radioactivity used approx. 5 μCi [^{3}H]diisopropylfluorophosphate (except where noted) plus enough unlabeled diisopropylfluorophosphate to constitute a 1000-fold mol excess. The excess was calculated assuming an average molecular weight of 300 000 for Factor V or Factor Va and that all protein in the sample might be coagulation factor. A molecular weight of 184.2 was used for [^{3}H]diisopropylfluorophosphate. The diisopropylfluorophosphate sample was tested for its activity against thrombin, a known serine protease, as well as against the Factor V activator from Russell's viper venom. The thrombin activity was totally inhibited as expected, whereas the venom activator under similar conditions was not affected. It is noteworthy that simple dilution of a venom activator and diisopropylfluorophosphate mixture indicated an apparent inhibition of the activator. However, if the diisopropylfluorophosphate/venom activator mixture was dialyzed against Michaelis-Ca^{2+} buffer*, complete recovery of the venom activator activity was achieved.

Procedures for the isolation, purification and assay of Factor V from bovine plasma and for its activation by thrombin or venom activator were those outlined previously by Smith and Hanahan [4]. The latter publication outlines a 2400-fold purification from starting plasma. Clotting activity was measured in Ortho units, the amount of Factor V activity contained in 1 ml of pooled, citrated human plasma. Over ten comparable preparations of Factor V from whole plasma were used in compiling this report. Assay of antithrombin III activity by Factor Xa inhibition was done according to Yin and Wessler [5]. Samples for radioactivity measurements were dispersed in 0.4% PPO in methanol/dioxane (2:3 v/v) and counted in a Beckman LS-230 scintillation unit (60% efficiency). Thrombin was assayed by a Mechrolab clot timer using greater than 95% clottable fibrinogen.

Factor V or Factor Va^T (Factor V activated by thrombin) was also incubated for 2 h with Michaelis-Ca^{2+} buffer* in the presence of 5 μCi [^{3}H]-Diisopropylfluorophosphate and applied without further treatment to a Sephadex G-200 column and eluted with the same buffer used for incubation. Controls of Factor V or Factor Va^T containing the same level of activity, but with no diisopropylfluorophosphate present, were treated in the same manner as described above. Usually a period of 18—24 h elapsed from the time of application of a control or diisopropylfluorophosphate-treated sample to a column and the assay for Factor V coagulant activity and radioactivity in each eluted fraction.

*Michaelis-Ca^{2+} buffer: 0.026 M sodium acetate/0.026 M sodium acetate·$3H_2O$/0.026 M sodium diethylbarbiturate/0.108 M NaCl/25 mM $CaCl_2$, adjusted with HCl to pH 7.35.

Analytical polyacrylamide gels, containing 4% total acrylamide and 6.25% bis-acrylamide were prepared in 0.5 × 10 cm tubes according to the method of Davis [6]. To these gels 200 μl Factor V (containing 1.8 mg/ml (A_{280}) extracted by the QAE-cellulose step of our procedure [4] was applied. Subsequent to electrophoresis, these gels were stained with Coomassie Brilliant Blue R. After destaining, these gels were scanned at 660 nm in a Gilford Model 240 spectrophotometer with Model S-2410 linear scanner. The gels were then frozen and sliced by razor blade into 2-mm sections. Each section was digested overnight in 1 ml 95% Protosol at 55°C. After cooling, 9 ml scintillation fluid was added to the digest and each vial was counted. Factor V activity was eluted from duplicate gels by incubating a 2 mm gel slice at 4°C in 0.4 ml Michaelis-Ca^{2+} buffer overnight and using 0.1 ml of this mixture for clotting assay on the following morning. Migration distance of each band was determined by measurement from the top of the gel and made relative to the distance traveled by Bromophenol Blue tracking dye.

The influence of antithrombin III and antithrombin III heparin mixtures on Factor V or Factor Va^T activity was followed in a manner similar to that described in the diisopropylfluorophosphate experiments above. Subsequent to incubation at 4°C, each sample was then placed on a Sephadex G-200 column as described above and the column eluted in the usual manner. Each fraction was assayed for its antithrombin III activity and for Factor V or Factor Va^T activity.

The data presented show clearly that bovine plasma Factor V, as well as the activated forms, Factor Va^T or Factor Va^V, (Factor V activated by venom activator) are not affected by diisopropylfluorophosphate. After removal of more than 99% radiolabeled diisopropylfluorophosphate (Table I) the remaining [^{3}H]diisopropylfluorophosphate can then be separated from Factor Va^V activity by column chromatography (Fig. 1A). If diisopropylfluorophosphate reacted in a ratio of 1 mol diisopropylfluorophosphate to 1 mol Factor Va^V, at least 2000 cpm should have been associated with column protein, but this was not observed (see Table I cpm). Similar results showing complete separation of protein and radiolabel on Sephadex G-200

TABLE I

ACTIVITY OF FACTOR V, FACTOR Va^T AND FACTOR Va^V IN THE PRESENCE OR ABSENCE OF DIISOPROPYLFLUOROPHOSHPATE

A 1000-fold excess of [^{3}H]diisopropylfluorophosphate was used with Factor V or Factor Va. A 1:1000 (v/v) ratio between sample and dialyzing buffer (0.2 M Tris acetate, (pH 7.5)/10% glycerol/50 mM $CaCl_2$) was maintained for 12 h with buffer changes every 4 h.

Sample condition*	[^{3}H]DFP added (cpm)	Radioactivity removed during dialysis, (% of added [^{3}H] DFP)			% of initial activity remaining in sample after 12 h dialysis	
		1st change	2nd change	3rd change	Clotting activity	Radioactivity
Factor V alone	—	—	—	—	100	—
Factor V + [^{3}H]DFP	$4.48 \circ 10^6$	84	11	1	100	0.4
Factor Va^T	—	—	—	—	69	—
Factor Va^T + [^{3}H]DFP	$2.5 \circ 10^6$	99	Trace	Trace	69	0.1
Factor Va^V	—	—	—	—	99	—
Factor Va^V + [^{3}H]DFP	$8 \circ 10^6$	99	Trace	Trace	105	0.01

*Each sample of Factor V contained 96 Ortho units; Factor Va^T contained 144 Ortho units; and Factor Va^V contained 648 Ortho units.

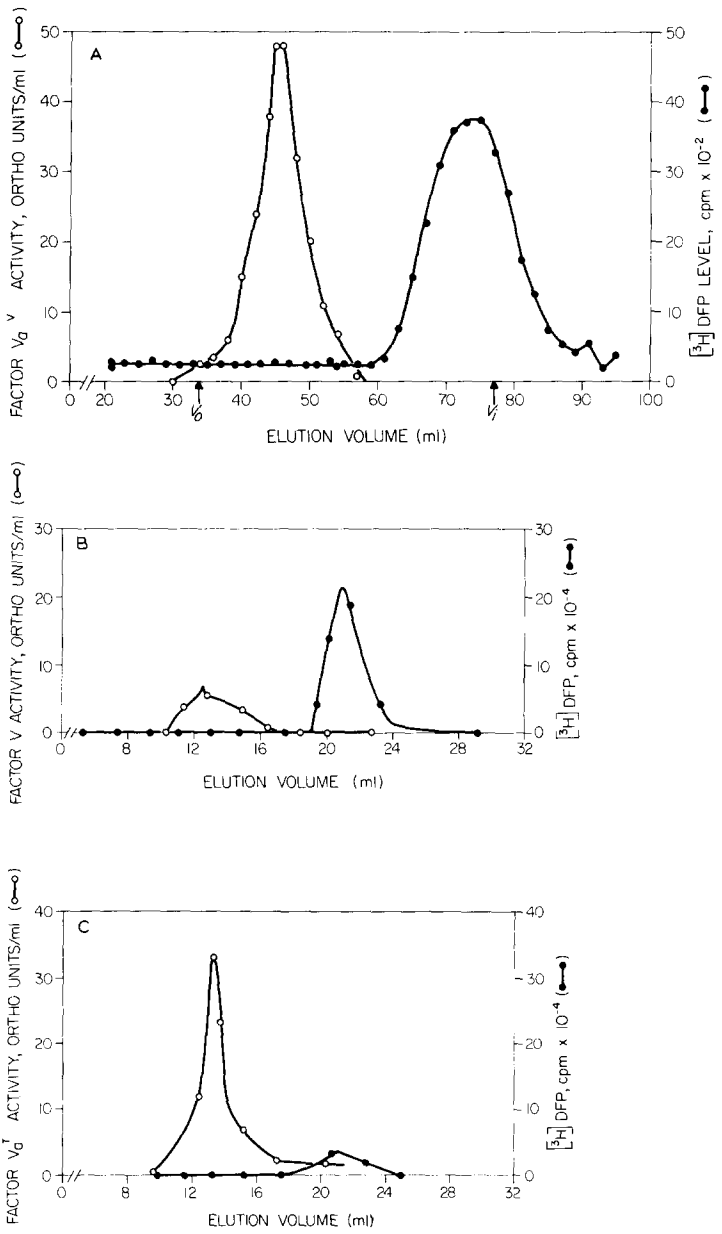

Fig. 1. Behavior of Factor V, Va^T and Va^V with diisopropylfluorophosphate (DFP) on Sephadex G-200 column chromatography. (A) Factor Va^V. Factor V, activated 6.2-fold by addition of 44 μg venom activator in 1.5 ml 0.2 M Tris acetate/10% glycerol/50 mM $CaCl_2$ (pH 7.5) was dialyzed immediately after addition of 0.27 μCi [^{3}H] diisopropylfluorophosphate in the same buffer for 4 h. 576 Ortho units of activity and 0.04 μCi were then applied to a 1.5 × 30 cm Sephadex G-200 column equilibrated and eluted at 4 ml/h with Michaelis-Ca^{2+} buffer (pH 7.35). 1 ml fractions of this buffer were eluted from the column and assayed immediately. 72% clotting activity was recovered. o—o, Factor Va^V activity in Ortho units/ml; ●—●, cpm [^{3}H] diisopropylfluorophosphate × 10^{-2}. (B) Factor V. After concentration by vacuum ultrafiltration with a Millipore immersible separator to 0.6 ml and incubation 2 h at room temperature with 5 μCi [^{3}H] diisopropylfluorophosphate, 141 Ortho units Factor V were applied to a 0.9 × 30 cm column of Sephadex G-200. The column was equilibrated overnight at a flow rate of 3.75 ml/h and eluted with Michaelis-Ca^{2+} buffer (pH 7.35) at the same rate.

were obtained with Factor Va^T or with native Factor V (unpublished data). Furthermore, biological activity of Factor V, Factor Va^T or Factor Va^V which was retained during dialysis and eluted from a column was not experimentally different from control samples treated identically but not containing diisopropylfluorophosphate.

Diisopropylfluorophosphate-treated samples incubated for 2 h without dialysis were also chromatographed on Sephadex G-200 (Fig. 1B and C). Biological activity appeared at the void volume and radiolabeled diisopropylfluorophosphate was at the inclusion volume. By this procedure, controls without diisopropylfluorophosphate lost the same amount of biological activity. The activity which was lost in control and treated samples was always equal if both samples were run simultaneously or in sequence.

Additional proof that diisopropylfluorophosphate did not interact with Factor V or Factor Va^T was obtained in the following experiment. Factor V (340 μg protein; 2.2 Ortho units) or Factor Va^T (340 μg protein; 24 Ortho units) was incubated with [^{3}H]diisopropylfluorophosphate and then examined by gel electrophoresis. Less than 5% coagulant activity was recovered as Factor V and less than 50% as Factor Va^T. Factor V activity was localized in one band but Factor Va activity diffused within the gels. Of importance, no radioactivity was associated with any portion of the gel. Nearly 75% of the radioactivity was removed from the gel during destaining, with the remainder lost initially into the upper chamber buffer of the electrophoresis apparatus.

To determine if an in vivo inhibitor of serine proteases in blood coagulation could be effective with Factor V or Factor Va^T, column chromatography with antithrombin III was also performed. Antithrombin III (0.165 mg/ml final concentration), either alone or as a complex with heparin (0.05 mg/ml final concentration) failed to bind Factor V or Factor Va^T. When complexed with antithrombin III·heparin, 80% of native Factor V activity was recovered but only 35% clotting activity was recovered with Factor Va^T under the same condition. In order to determine if any of the lost activity was the result of antithrombin III·heparin inhibition or due to inherent instability, Factor Va^T was incubated for 6 h at 4°C with a measured amount of antithrombin III·heparin complex. An amount of purified thrombin equivalent to (or required to neutralize) the antithrombin III·heparin complex was added to the Factor Va^T sample and the Factor Va^T activity determined after 20 min. If antithrombin III·heparin had complexed irreversibly with Factor Va^T, thrombin could not be totally neutralized by less than an equivalent amount of reactive antithrombin III·heparin. Factor Va^T activity would presumably be less than in the control because of the irreversible antithrombin III·heparin inhibition. Using triplicate samples, no thrombin activity was detected and Factor Va^T activity was experimentally identical to

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0.5-ml fractions were collected and contained 62% of the applied clotting activity. ○–○, Factor V activity in Ortho units/ml; ●–●, cpm [^{3}H]diisopropylfluorophosphate × 10^{-4}. (C) Factor Va^T. Factor Va prepared by addition of 2 μg purified thrombin to 24 Ortho units of Factor V in 0.6 ml were applied after incubation for 2 h with 5 μCi [^{3}H]diisopropylfluorophosphate at room temperature. 145 Ortho units were applied and eluted in a 0.9 × 30 cm column as above with 70% recovery of clotting activity. ○–○, Factor Va^T activity in Ortho units/ml; ●–●, cpm [^{3}H]diisopropylfluorophosphate × 10^{-4}.

the control. Therefore, we concluded that antithrombin III·heparin complex has no irreversible interaction with Factor Va^T at 4°C.

This study shows that in the presence of excesses of diisopropylfluorophosphate and antithrombin III·heparin, no complexes with Factor V, Factor Va^T or Factor Va^V were detected. Hence, based on this criteria, it is apparent that Factor V and Factor $Va^{T/V}$ are not serine proteases and cannot be compared on the basis of protease activity with other coagulant proteins. Thus, with the finding that bovine plasma Factor VIIIa, generated from bovine plasma Factor VIII by thrombin action, is inhibited by diisopropylfluorophosphate [2], Factor V(Va) remains unique in the coagulation scheme of reactions in not possessing any (protease) enzymatic activity.

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