BBA 63441

Inhibition of thrombin by sarin

Inhibition of thrombin (EC 3.4.4.13) esterase activity by isopropyl methylphosphonofluoridate (sarin, 50% with 0.1 mM) was reported by SHUSTER *et al.*[1]. MOUNTER *et al.*[2] found less than 30% inhibition of thrombin clotting with 1.0 mM sarin. Inhibition conditions differed, but not sufficiently to account for the disparity in the results. Both studies used a commercial, biologically activated prothrombin, noted for instability[3] due to contaminant inhibitors and esterases[4].

Sarin inhibition of crude and partially purified[5] thrombins was investigated to establish if the enzyme is sensitive to the organophosphonate and if clotting and esterase activities respond identically to the inhibitor. Reactivation of sarin-inhibited thrombin was also studied.

Crude bovine thrombin (Parke, Davis and Co.) was stored at −20° in aliquots with 500 units/ml in 0.15 M KCl. Partially purified thrombin was prepared by the method of RASMUSSEN[5] (10 000 units of crude thrombin in 2 ml initial buffer to a 25 cm × 1 cm bed of Amberlite CG-50 Type 2, Fisher). A 20-fold purification was achieved with 90% activity, and aliquots were rapidly frozen and stored at −20°. Fibrinogen (bovine Fraction I, Fisher Chemical Co.; 10 mg/ml 0.05 N sodium phosphate, pH 6.7) was further purified by centrifugation of the cryoprecipitate[6] after standing overnight at 4°. Fresh preparations were used each day. Clotting times were measured in 10 mm × 75 mm glass tubes at 25°. With a micropipette, 0.02 ml thrombin (diluted to give clotting times between 25 and 50 sec, 5–10 Parke–Davis units/ml) was transferred to 0.28 ml fibrinogen preparation, the tube flicked, and the time to the first visible opacity recorded. Duplicates agreed to within 5%. There was some variation among different lots of thrombin. Log clotting time *versus* log thrombin concentration was linear for daily standard curves. Hydrolysis of *p*-tosyl-L-arginine methyl ester (TAME, Mann Research Laboratories) was determined by a titrimetric procedure similar to that of EHRENPREIS AND SCHERAGA[3]. A Radiometer TTT 1-C titrator with SBR2/SBU1/TTA3 assembly was employed, with reactions run at 37° in the absence of additional buffer. Standard base (0.01 M NaOH) was made up fresh each day. TAME (0.02 M in 0.15 M KCl) was adjusted to pH 8.0, and 3.0 ml were pipetted into the reaction vessel, with temperature equilibrated by rapid magnetic stirring. The spontaneous hydrolysis rate (0.04–0.05 μmole · min^{-1}) was recorded and subtracted from the measured rate with the enzyme. Thrombin (0.100 ml, about 5 Parke–Davis units) was delivered and the addition of base *versus* time continuously recorded (to 0.900 μmole · min^{-1}).

For inhibition, sarin (98% pure by carbon and hydrogen analysis; from Dr. R. I. Ellin, Edgewood Arsenal) was freshly opened for each experiment, diluted in 0.3 M KCl and added immediately to thrombin, which had been dialyzed overnight against 0.15 M KCl (crude) or 0.3 M KCl (partially purified)*. Various concentrations of sarin in 0.015 M Tris (pH 8.0) for 60 min at 25° were used and values from four to

Abbreviations: TAME, *p*-tosyl-L-arginine methyl ester; 2-PAM Cl, 2-pyridine aldoxime methochloride.

* Partially purified thrombin was stable for 2 weeks at 25° in the buffer as eluted, but dialysis against 0.3 M KCl resulted in 15% loss of activity.

eight separate experiments for each curve were in close agreement. Values reported are from a single, representative vial of sarin. Both esterase and clotting activities were inhibited, the pH optima being 8. At 37° the rate of inhibition increased, but the thrombin was less stable. Between 30 and 60 min, inhibition decreased sharply. Fig. 1 is a plot of inhibition of crude thrombin as % uninhibited control *versus* the negative log of sarin concentration. Clotting and esterase activities differ in sensitivity—at 3.0 μM sarin esterase was fully active while clotting was inhibited greater than 20%. These data are in fair agreement with SHUSTER *et al.*[1], but differ from the results of MOUNTER *et al.*[2]. For partially purified thrombin (Fig. 2) clotting and esterase activities were essentially equally inhibited by each concentration of sarin (0.3–300 μM).

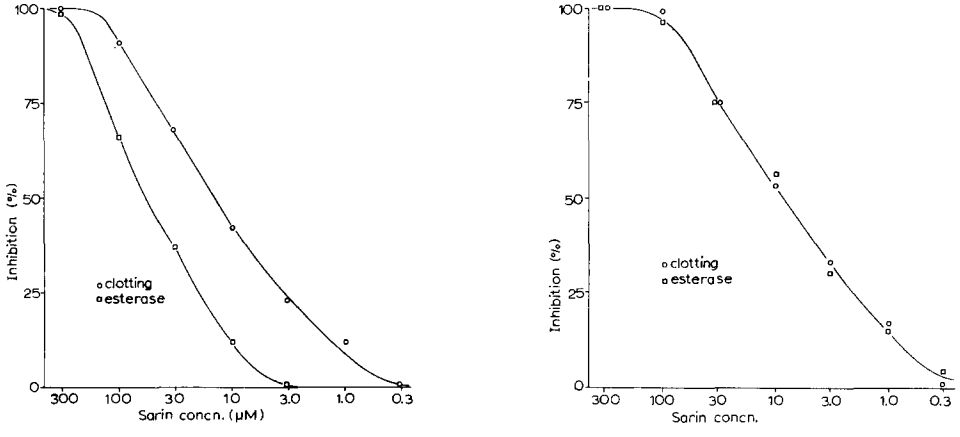

Fig. 1. Inhibition of crude thrombin at different concentrations of sarin, pH 8.0, 25°; 60 min.
Fig. 2. Inhibition of partially purified thrombin at different concentrations of sarin, pH 8.0, 25°, 60 min.

GLADNER AND LAKI[7] and MILLER AND VAN VUNAKIS[8] first demonstrated thrombin inhibition by the organophosphate, DFP. Another study indicated that DFP inhibited the clotting activity of crude thrombin more readily than the esterase activity[9]. An esterase contaminant in the crude preparation that would hydrolyze TAME yet be less sensitive to DFP and have no effect upon fibrinogen would account for such a difference. Activated Factor X (X_a, thrombokinase) could well be the contaminant in question as it has been isolated from crude thrombin[10,11]. In a highly purified form, X_a has been found to hydrolyze TAME yet it could only be inhibited by high (10 mM) concentrations of DFP[12]. The same esterase would account for the differences in sarin inhibition of the crude and partially purified thrombins observed in the present study.

Partially purified thrombin, inhibited by 1.0 mM sarin at pH 7 (99% inhibited in 15 min) could be reactivated in the eluted phosphate buffer at pH 7 (less so at pH 8) either spontaneously or with 2-pyridinealdoxime methochloride (2-PAM Cl, Ayerst Laboratories). From a 2.0 M stock solution the 2-PAM Cl was adjusted to pH 7.5 immediately prior to use. The oxime had a moderate inhibitory effect on uninhibited thrombin controls. Reactivation results are summarized in Table I.

Esterase activities exactly paralleled the clotting reactivation. Adding 2-PAM Cl 24 h after the sarin resulted in somewhat slower reactivation, although a visible protein precipitate had formed in the inhibited thrombin solutions by 24 h.

Partial reactivation of DFP-inhibited thrombin by 0.6 M hydroxylamine was demonstrated by GLADNER AND LAKI[7]. SHUSTER *et al.*[1] could not reactivate the sarin-inhibited thrombin using pyridine-2-aldoxime methiodide, although their conditions were pH 9 and an oxime concentration of 0.02 M. In the present study complete reactivation of sarin-inhibited thrombin with 2-PAM Cl was achieved using higher concentrations of the oxime and a lower pH. Spontaneous reactivation was also noted and the 17% activity at 96 h compares favorably with the 23–36% spontaneous reactivation for the same period reported by GREEN AND NICHOLLS[13] for chymotrypsin. The reactivation rate can be calculated[13] from the data in Table I and for 0.3 M 2-PAM Cl the rate is 1.0 $l \cdot moles^{-1} \cdot h^{-1}$ which is quite close to 0.7 $l \cdot moles^{-1} \cdot h^{-1}$ obtained by reactivating sarin-inhibited α-chymotrypsin with 2-PAM Cl under the same conditions (author, unpublished experiments).

Isopropyl methylphosphonofluoridate (sarin) inhibits the clotting and esterase

TABLE I

REACTIVATION OF SARIN-INHIBITED PARTIALLY PURIFIED THROMBIN

Oxime added 20 min after 1.0 mM sarin to partially purified thrombin, pH 7.0, 25°. Clotting activity as percentage of uninhibited control.

*2-PAM Cl concn.** (*M*)	*Clotting activity*				
	Incubation time after oxime addition (h):				
	3	*6*	*24*	*48*	*96*
None (spontaneous)	0		8	14	17
0.01	—		16	—	—
0.1	—	48	94	95	—
0.3	57	85	100	100	—
0.3*	—	50	90	—	—

* Oxime added 24 h after sarin.

activities of bovine thrombin. With a crude thrombin preparation, clotting was more sensitive to inhibition than was esterase activity. Upon partial purification, ester hydrolysis and fibrinogen clotting became equally sensitive to inhibition by sarin. These differences can be ascribed to the removal of a contaminant enzyme, which would hydrolyze the ester but have a different sensitivity to the inhibitor. Spontaneous reactivation of sarin-inhibited thrombin occurs. With 2-pyridinealdoxime methochloride complete reactivation can be demonstrated. Inhibited thrombin does not lose its ability to be reactivated by the oxime.

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1 J. SHUSTER, J. F. SCAIFE AND G. A. GRANT, *Can. J. Biochem. Physiol.*, 37 (1959) 895.
2 L. A. MOUNTER, B. A. SHIPLEY AND M. E. MOUNTER, *J. Biol. Chem.*, 238 (1963) 1979.
3 S. EHRENPREIS AND H. A. SCHERAGA, *J. Biol. Chem.*, 227 (1957) 1043.
4 D. J. BAUGHMAN AND D. F. WAUGH, *J. Biol. Chem.*, 242 (1967) 5252.
5 P. S. RASMUSSEN, *Biochim. Biophys. Acta*, 16 (1955) 157.
6 K. LAKI, *Arch. Biochem. Biophys.*, 32 (1951) 317.
7 J. A. GLADNER AND K. LAKI, *Arch. Biochem. Biophys.*, 62 (1956) 501.
8 K. D. MILLER AND H. VAN VUNAKIS, *J. Biol. Chem.*, 223 (1956) 227.
9 L. A. MOUNTER, K. D. TUCK, H. C. ALEXANDER AND L. T. H. DIEN, *J. Biol. Chem.*, 226 (1957) 873.
10 D. M. KERWIN AND J. H. MILSTONE, *Thromb. Diath. Haemorrhag.*, 17 (1967) 247.
11 E. T. YIN AND S. WESSLER, *J. Biol. Chem.*, 243 (1968) 112.
12 C. M. JACKSON AND D. J. HANAHAN, *Biochemistry*, 7 (1968) 4506.
13 A. L. GREEN AND J. D. NICHOLLS, *Biochem. J.*, 72 (1959) 70.

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