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Reversible Modulation of Human Factor Xa Activity with Phosphonate Esters: Media Effects

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Abstract—Enantiomers of 4-nitrophenyl 4-X-phenacyl methylphosphonate esters (X = H, PMN; CH_3 and CH_3O) inactivate human factor Xa with rate constants 8–86 M^{-1} s^{-1} at pH 6.75 in 0.025 M Hepes buffer, 0.15 M NaCl and 2 mM $CaCl_2$ at 7.0 ± 0.1 °C. The stereoselectivity of the inactivation of factor Xa is 2–10 and favors the levorotatory enantiomers. The pH-dependence of inactivation of factor Xa by (−)-PMN is sigmoidal and consistent with the participation of a catalytic residue with a pK_a of 6.2 ± 0.1. Factor Xa reactivates from its phosphonyl adducts through a self-catalyzed intramolecular reaction, which is much influenced by the presence of phospholipids. The rate of reactivation in the absence of phospholipids is not pH dependent at pH < 9, but it increases very much at pH > 9. In the presence of phospholipids, the pH dependence of the rate constant for reactivation is sigmoidal in the pH 6.5–10.3 range and levels off at pH > 9 indicating that the enzyme catalyzes its reactivation. The kinetic pK_a for the recovery of factor Xa from its adducts with the PMNs is in the range of 6.7–8.1 and is consistent with the participation of the catalytic His57 in the reactivation process.

Introduction

Factor Xa (EC 3.4.21.6 or Xaα) is a glycosylated serine protease, synthesized at the convergence of the intrinsic and extrinsic pathways of the blood coagulation cascade. Factor Xa plays the very important role of cleaving prothrombin at two sites, Arg274-Thr275 and Arg323-Ile324 (prothrombin numbering) generating α-thrombin.[1] Factor Xa activates also factor VII, as a form of positive feedback in the blood coagulation cascade. The activation process is very similar to the activation of pancreatic proteases, the only difference being that it involves more components and is localized on a membrane surface.[2]

The complex, prothrombinase, is assembled on phospholipid membranes, modeled by mixtures of phosphatidyl serine (25% mol) and phosphatidyl choline (75%), and requires Ca^{2+} for proper binding of prothrombin (substrate), factor Va (cofactor) and factor Xa (enzyme). Experiments proved that the configuration of the polar head group of serine in phosphatidyl serine (PS), PLS or PDS, is of major importance in providing optimal binding of factor Va and Xa and that PLS favors binding 5-fold over PDS. It was also revealed that factor Va has a recognition site for PLS that does not require Ca^{2+}.[3]

Factor Xa consists of a light chain (139 amino acid residues) and a heavy chain (251 amino acid residues) linked together by a disulfide bond.[4–6] Factor Xa has a modular structure characteristic of many of the blood clotting cascade proteases.[7] X-ray structure data[8,9] show that the different domains of factor Xa can have various orientations relative to each other, which indicate high flexibility and large conformational changes. Also, upon binding of factor Va, the distance of the active site to the membrane changes significantly.[10]

Abbreviations: 7-AMC, 7-amino(amido) 4-methylcoumarin; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; Boc, tert-butoxycarbonyl; C6PS, 1,2-dicaproyl-sn-glycero-3-[phospho-L-serine}; CAPS, 3-{cyclohexylamino]-1-propanesulfonic acid; CHES, 2-[*N*-cyclohexylamino] ethanesulfonic acid; Chiracel-OJ, cellulose tri-4-methyl-benzoate coated silica; EDTA, ethylenediaminetetraacetic acid; HPLC, high pressure liquid chromatography; HEPES, *N*-[2-hydroxyethyl} piperazine-*N*′-[2-ethanesulfoic acid]; LUV, large umlamellar vesicles; MES, 2-[*N*-morpholino] ethanesulfonic acid; PCh, phosphatidyl choline; PEG-4000, polyethylene glycol (MW≈4000); PMN, 4-*H*-phenacyl 4-nitrophenyl methylphosphonate; PMNs, 4-substituted-phenacyl 4-nitrophenyl methylphosphonates; PS, phosphatidyl serine; TLC, thin layer chromatography; Tris, tris-(hydroxymethyl)-aminomethane.

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The active site is relatively accessible and exposed as in trypsin.[8] The catalytic triad is formed by Ser195, His57 and Asp102 (chymotrypsin numbering system) and is located in the heavy chain. Asp189 is at the bottom of the specificity pocket (S1) and forms a salt bridge with Arg/Lys residues (P1) of substrates. The S2 site is occupied by Tyr99. Its mutation,[11] Tyr99Thr, proved that this site is critical for substrate specificity. Chromogenic substrates having Gly in the P2 position were cleaved up to 137-fold slower by a Tyr99Thr/Gln192Glu double mutant than by the native enzyme.[11] However, the side chain of Tyr99 is flexible and a tight-binding ligand could expand this binding site by moving Tyr99.[9]

Thrombin, factor Xa, and factor IXa can cause the same thrombogenic effect in molar equivalents of 1:0.1:0.016, respectively. Thus, factor Xa would be a rational target of inhibition if it were not highly concentrated on membrane surfaces, which then requires high concentrations or tight-binding inhibitors. The structural framework for inhibition of factor Xa has recently been reported.[12,13]

Although there are a large number of synthetic low molecular weight inhibitors designed for thrombin, much fewer have been designed for factor Xa.[12–16] Substituted 4-amidinophenyl benzoates were studied as potential reversible inhibitors of factor Xa. They acylate factor Xa and the acyl-factor Xa reactivates in time with $t_{1/2} = 6$–100 min[15,16] suggesting that acyl-factor Xa could be used as a prodrug for a timed release of active factor Xa.

Temporal chemical modification of an active site has been one of the research interests in this laboratory. Phosphonate ester inhibitors have been studied as candidates because they provide greater diversity in physicochemical properties than the corresponding carboxyl ester due to higher valence states in P than in C. The physiologic properties of the anionic monophosphonate ester products are likely to be harmless. Herein, we report reversible modulation of factor Xa activity by 4-nitrophenyl 4-substituted phenacyl methylphosphonate ester (PMN; 4-CH_3-PMN; 4-CH_3O-PMN) inhibitors. The PMNs have a chiral center at the phosphorus atom. We have shown[17,18] that serine proteases, particularly trypsin and chymotrypsin, prefer the levorotatory enantiomer over the dextrorotatory enantiomer. Since factor Xa is a trypsin-like enzyme, it was expected that it would be inhibited by the PMN group of inhibitors and that it would prefer the levorotatory enantiomers. The enantioselectivity of factor Xa for the three derivatives of PMNs has been found to be smaller than for related enzymes, within two and ten, favoring the levorotatory enantiomers. As in the case of trypsin, chymotrypsin, t-PA and thrombin, the inactivation of factor Xa by PMNs is reversible due to carbonyl-participation in the dephosphonylation step,[17–20] although the reactivation process is enzyme catalyzed only in the presence of phospholipids. The results of these studies are relevant to a potential medical use of temporarily phosphonylated factor Xa.

Results

Inhibitory potency of PMNs towards factor Xa

Enantiomers of PMN, 4-CH_3-PMN and 4-CH_3O-PMN, were used as reversible inhibitors of factor Xa. The following equation describes the reaction:

$$E + I \overset{K_i}{\rightleftarrows} E{\cdot}I \overset{k_i}{\rightarrow} E_{inh} + p - \text{nitrophenol} \quad (1)$$

The initial concentration of the inhibitor $[I_0]$ was at least 30-fold larger than the concentration of the enzyme, $[E_0]$, thus $[I_0]$ can be considered constant during the first 2–4 min of the reaction. The inhibitory capacity of the six compounds was studied at pH 6.75, where the buffer-catalyzed hydrolysis of the inhibitor can be neglected. Expressing the observed rate constant in terms of Michaelis–Menten parameters, K_i and k_i, and integrating the resulting differential equation between the limits $t = 0$ and t yields:

$$\ln\frac{[E_0]}{[E]} = \frac{k_i[I_0]}{K_i + [I_0]}t \quad (2)$$

The enzyme concentration is linearly proportional to the initial rate of substrate hydrolysis and, thus, to the release of 7-AMC from the fluorogenic substrate used in the enzyme assay. Enzyme assays were run after 2, 3 or 4 min incubation with an inhibitor, depending on the potency of the inhibitor. The inverted form of eq (2) was used for data fitting;

$$\frac{1}{\ln\frac{v_0}{v}} = \frac{1}{k_i t} + \frac{K_i}{k_i t}\frac{1}{[I_0]} \quad (3)$$

where t is the chosen reaction time held constant throughout a set of experiments. Values for $1/(\ln v_0/v)$ versus $1/I_0$ were plotted. As predicted by eq (3), the inverse of the intercept of the line gives k_i and the inverse of the slope gives k_i/K_i. The largest errors are in defining the intercept. The results for inactivation of factor Xa by PMNs are presented in Table 1.

pH-dependence of the inactivation of factor Xa by (−)-PMN

The pH-dependence of the inactivation of factor Xa using (−)-PMN was studied in the range pH 5.7–8.0. Since buffer-catalyzed hydrolysis of the PMNs increases with pH, correction for $[I_0]$ had to be applied at pH

Table 1. Inactivation of factor Xa by PMNs at pH 6.75 in 0.025 M HEPES buffer, 0.15 M NaCl, 2 mM $CaCl_2$, at 7.0 ± 0.1 °C[a]

4-X-PMN	k_i/K_i (M^{-1} s^{-1})	10^2 k_i (s^{-1})	10^4 K_i (M)
-H(−)	82 ± 6	0.8 ± 0.1	1.0 ± 0.1
-H(+)	16 ± 4	0.8 ± 0.2	4.6 ± 1.0
-CH_3(−)	86 ± 13	0.5 ± 0.1	0.6 ± 0.1
-CH_3(+)	8 ± 2	0.5 ± 0.1	6.6 ± 1.8
-OCH_3(−)	20 ± 1	0.7 ± 0.1	3.5 ± 0.3
-OCH_3(+)	16 ± 7	0.5 ± 0.1	3.0 ± 0.7

[a]The rate constants are the mean values of two sets of measurements.

> 7.3. The buffer-catalyzed hydrolysis of PMNs follows first-order kinetics, so the time-dependent inhibitor concentration can be expressed by $[I_0]\exp(-k_h t)$, where k_h is the hydrolysis rate constant. This parameter, k_h, was determined from independent spectrophotometric measurements, in the buffers used for inhibition, at 7.0 ± 0.1 °C. When the correction is applied, another non-linear equation is derived:

$$\ln\frac{v_0}{v} = \frac{k_i}{k_h}\ln\frac{K_i + [I_0]}{K_i + [I_0]e^{-k_h t}} \quad (4)$$

The second-order rate constant for inhibition, k_i/K_i, of factor Xa by (−)-PMN at various pH was calculated and the obtained profile is presented in Figure 1. The second-order rate constants versus pH were fit to eq (5);

$$k = \frac{k_{lim1}K_a}{K_a + [H^+]} + k_{lim2} \quad (5)$$

The pK_a of the catalytic residue involved directly in inhibition of factor Xa is 6.21 ± 0.06 and the upper limit of the rate constant is $k_{lim1} = 114 \pm 5\ M^{-1}\ s^{-1}$.

Reactivation of factor Xa from adducts formed with PMNs

Inhibition of factor Xa by PMNs proved to be reversible. Control experiments were carried out to determine the denaturation of the enzyme during lengthy experiments (up to 8 h). The general equation describing the reactivation process is:

$$E_{inh} \overset{k_r}{\rightarrow} E \overset{k_d}{\rightarrow} E_{denatured} \quad (6)$$

where E_{inh} represents the inhibited enzyme, E is the reactivated enzyme and $E_{denatured}$ is the denatured enzyme. The rate constants which characterize these processes are k_r for reactivation and k_d for denaturation. The concentration of the recovering enzyme is expressed by the following equation:

$$[E] = [E_{inh}]k_r\frac{e^{-k_r t} - e^{-k_d t}}{k_d - k_r} + [E_0]e^{-k_d t} \quad (7)$$

where $[E_0]$ is the concentration of uninhibited enzyme at the beginning of the reactivation process. Reactivation was studied for adducts formed after inactivation of factor Xa by the individual enantiomers of the three compounds, PMN, 4-CH_3-PMN and 4-CH_3O-PMN in the 6.5–11.1 pH range. The pH-dependence profiles for the reactivation of factor Xa are presented in Figure 2. Surprisingly, they do not describe a sigmoidal curve. This indicates the absence or improper alignment (location) of the catalytic residue(s) which would facilitate reactivation under these experimental conditions. The rate constants seem to be dependent on the concentration of [OH^-] and [buffer]. The reactions were carried out at high salt concentration, 0.7 M NaCl, to minimize denaturation of the enzyme. In trial experiments, 2 mM $CaCl_2$ was added in the presence of 0.5 M NaCl, but this did not affect the rate of enzyme recovery. The analysis of data above pH 9 shows a steep slope approaching 2. This implies that besides the hydroxyl catalyzed reaction there is another component which accelerates hydrolysis at very high pH. In contrast, the reactivation of thrombin and other serine proteases studied earlier, give typical sigmoidal curves in the 6.2–9.8 pH range and a kinetic pK_a in the range of 7.2–8.0.[17,18,20]

Reactivation of factor Xa from (−)-4-CH_3-PMN inhibited nitro-factor Xa

Since reactivation of factor Xa from its adducts with (−)-PMNs occurred without apparent participation of a catalytically active residue in the pH 7–8.5 range, the hypothesis of Tyr99 participation seemed plausible. Tyr99 is very close to the active-site His57 and it seems

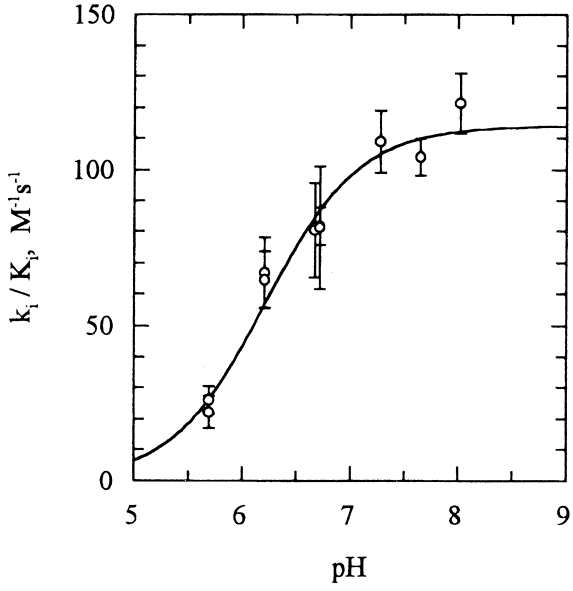


Figure 1. pH-dependence of the second-order rate constant, k_i/K_i, for the inactivation of factor Xa by (−)-PMN at 7.0 ± 0.1 °C.

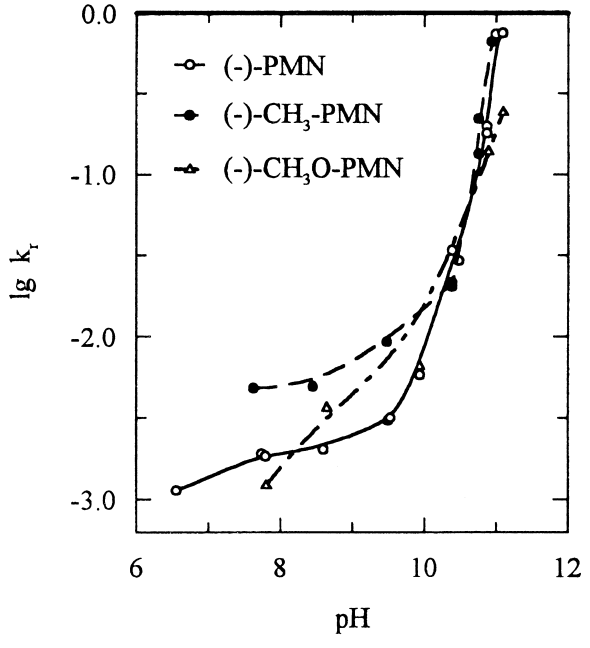


Figure 2. Log k_r versus pH profiles for the reactivation of factor Xa from its adducts with (−)- PMNs at 25.0 ± 0.1 °C.

from the X-ray structure that there is a $\pi-\pi$ interaction between the two side-chains. Nitration of this Tyr would lead to its ionization at pH < 10. Nitration proceeded as presented in the Experimental and the presence of nitro-factor Xa was detected by UV–vis spectroscopy. Reactivation of nitro-factor Xa from its adduct with (−)-4-CH_3-PMN has a similar pH dependence to the adduct with the native enzyme, yet an overall increase in the recovery rate is noticeable. Still the pH-rate profile is not sigmoidal, indicating that the ionized Tyr99 does not significantly influence the reactivation of the enzyme (Fig. 3).

Reactivation of factor Xa from adducts formed with (−)-4-CH_3-PMN in the presence of 1,2-dicaproyl-sn-glycero-3-[phospho-L-serine] (C6PS)

Inactivation of factor Xa by (−)-4-CH_3-PMN and reactivation in 0.35 mM C6PS[21] containing buffer was carried out as described in the Experimental. A wide pH range was covered and multiple runs were performed at some pH values. The experimental data are presented in Figure 4. An ionizing group with $pK_a = 7.2 \pm 0.1$ is clearly active in the catalytic recovery of factor Xa. The enzyme most probably adopts a different conformation upon binding C6PS and the active-site geometry changes enough to bring His57 into a favorable position for catalysis. As shown in Figure 4, the rates of reactivation level off at pH > 9, suggesting that the conformation of the enzyme is stabilized in the presence of C6PS.

Reactivation of factor Xa adducts in the presence of large unilamellar vesicles (LUV)

LUV were prepared as described in the Experimental. Reactivation of factor Xa was again monitored using a fluorogenic substrate. Multiple runs were performed at some pH values to assure that the data were reproducible. The rate constants for recovery from the adducts formed with (+)-PMNs and (−)-PMNs are presented in Figures 5 and 6, respectively. All pH profiles, except for the one for the recovery from (−)-PMN-inhibited factor Xa are sigmoidal consistent with an acid/base catalytically active residue, most probably His57. The pK_a values presented in Table 2 are slightly lower than pK_as of analogous reactions of thrombin.[20] We have no explanation for the curve obtained with (−)-PMN-inhibited factor Xa.

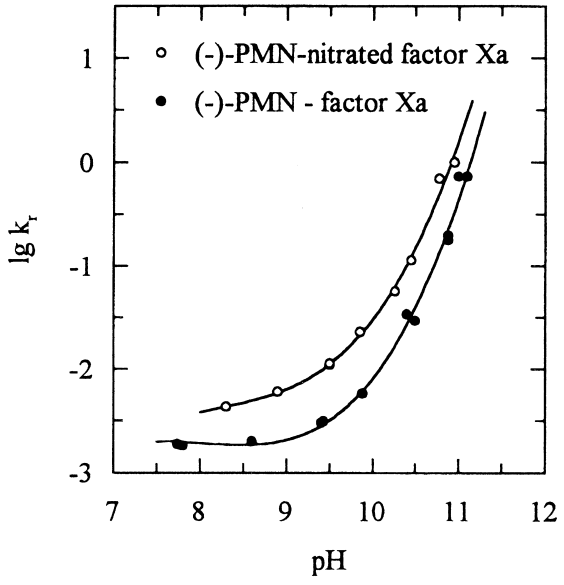


Figure 3. Log k_r versus pH profiles for the recovery of factor Xa and nitro-factor Xa from inactivation with (−)-CH_3-PMN at 25.0 ± 0.1 °C.

Reactivation of factor Xa adducts in the presence of LUV and factor Va

The natural substrate for factor Xa, prothrombin, is cleaved 10^5 times faster by the fully assembled prothrombinase complex than by factor Xa in solution.[22,23]

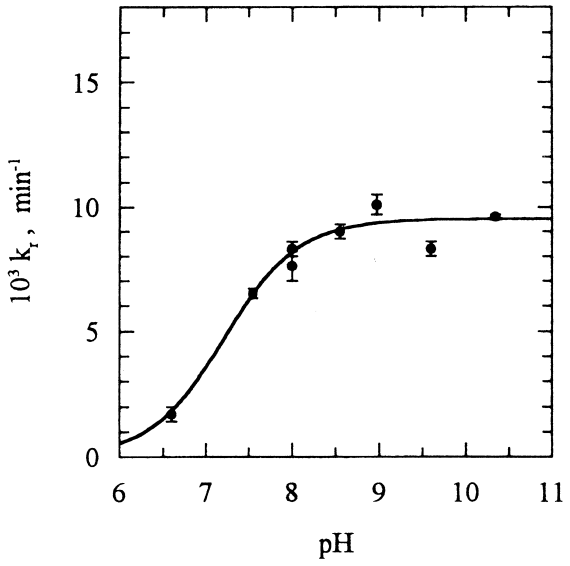


Figure 4. Reactivation of (−)-CH_3-PMN-inactivated factor Xa in the presence of C6PS at 25.0 ± 0.1 °C.

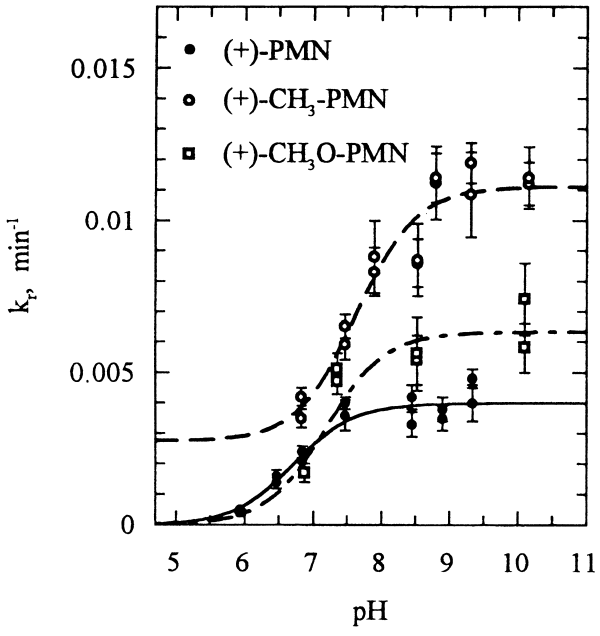


Figure 5. pH-dependence of the rate constants for reactivation of factor Xa from adducts formed with (+)-PMNs in the presence of LUV at 25.0 ± 0.1 °C.

Simulation of the prothrombinase complex was attempted by adding LUV and a stoichiometric amount of factor Va to the inhibited factor Xa. The rates of reactivation of factor Xa from the adducts are presented in Table 3. An analysis of the experimental data shows that the rates measured in the presence of LUV with and without factor Va in the mixture do not differ significantly. This could be due to the small size of the phosphonyl fragment linked to the enzyme. The fragment is accommodated at the active site and does not allow for interactions with the enzyme at remote, secondary, binding sites. The lack of difference in rate constants can also be due to the improper assembly of the prothrombinase complex.

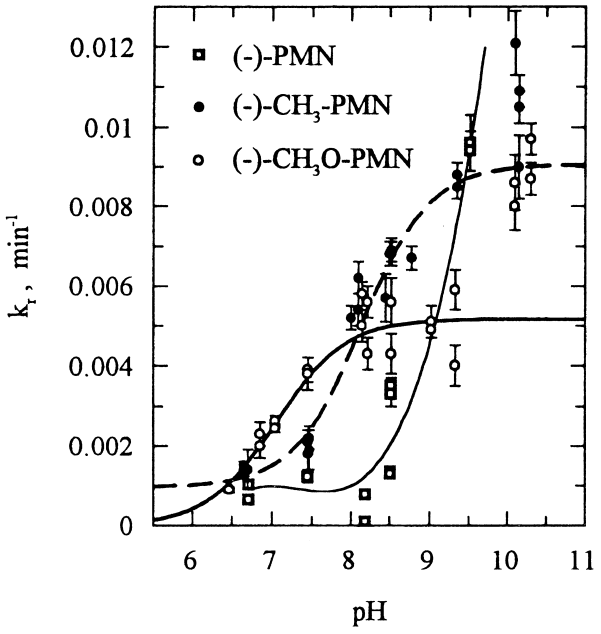


Fig.6. pH-dependence of the rate constants for reactivation of factor Xa from adducts formed with (−)-PMNs in the presence of LUV at 25 ± 0.1 °C.

Table 2. pH-independent rate constants and pK values for the reactivation of factor Xa from its adducts with PMNs in LUV containing buffer at 25.0 ± 0.1 °C

4-X-PMN	pK_a	$10^3\ k_{lim1}$ (min^{-1})	$10^3\ k_{lim2}$ (min^{-1})
-H(+)	6.7 ± 0.1	4.0 ± 0.2	—
-CH_3(−)	8.1 ± 0.1	9.1 ± 0.4	1.0 ± 0.2
-CH_3(+)	7.6 ± 0.2	11.1 ± 0.4	2.7 ± 0.7
-OCH_3(−)	7.1 ± 0.1	5.2 ± 0.2	—
-OCH_3(+)	7.2 ± 0.1	6.3 ± 0.5	—

Table 3. Rate constants for reactivation of factor Xa from its adducts in the presence of LUV, with and without factor Va, in 0.02 M Tris buffer, 2 mg/mL phospholipids, 0.15 M NaCl, 2 mM $CaCl_2$, and 0.1% PEG-4000 at 25.0 ± 0.1 °C

4-X-PMN	pH	$10^3\ k_r$ (min^{-1})	pH	$10^3\ k_r$ (min^{-1}), F Va
-H(−)	7.45	1.2 ± 0.1	7.60	0.61 ± 0.02
-H(+)	7.50	3.8 ± 0.5	7.60	3.1 ± 0.6
-CH_3(−)	7.47	2.0 ± 0.4	7.60	6.5 ± 0.5
-CH_3(+)	7.47	6.2 ± 0.5	7.60	6.4 ± 0.5

Discussion

Inactivation of factor Xa by PMNs

The PMNs are nearly as effective inactivators of factor Xa as of thrombin. The second-order rate constants for the inactivation of factor Xa are 8–86 $M^{-1}\ s^{-1}$; similar to the rate constants, k_i/K_i = 4–235 $M^{-1}\ s^{-1}$, for the inactivation of thrombin[20] but smaller than k_i/K_i = 470–750 $M^{-1}\ s^{-1}$, for t-PA [18] at neutral pH and 7 °C and k_i/K_i = 340–880 $M^{-1}\ s^{-1}$ for trypsin and $k_i/K_i = 3.7\times10^4$–$2.0\times10^5\ M^{-1}\ s^{-1}$ for chymotrypsin at 25 °C.[17] This is closely related to the nature of the specificity pocket and the access to the active-site. Chymotrypsin prefers substrates with aromatic side-chains in the P1 position, so it is no surprise that it presents the largest k_i/K_i in the reaction with PMNs. Thrombin prefers Arg or Lys as P1 residues, as does factor Xa, but has a large insertion loop (60a–60i) which makes the entrance to the active site narrower. Factor Xa presents the largest enantioselectivity, close to 10-fold, for 4-CH_3-PMN, and smallest, close to 1.3-fold, for 4-CH_3O-PMN.

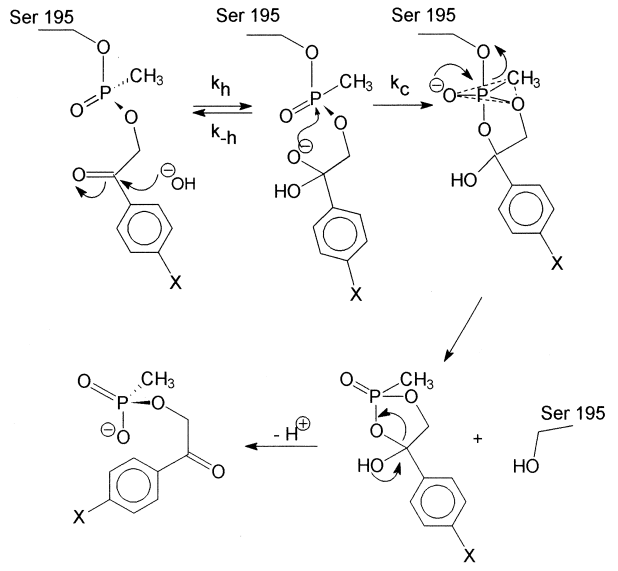
An analysis of the pH dependence of k_i/K_i for the inhibition of factor Xa by (−)-PMN shows that the kinetic pK_a of factor Xa is 6.21 ± 0.06. This is consistent with the fact that the protonation of a histidine residue, most probably His57, decreases the rate of inhibition in the pH range studied. In comparison, the inhibition of thrombin by (−)-PMN in 0.15 M NaCl gives pK_a = 8.0 ± 0.1 and is salt dependent. Such differences in pK for the inhibition of the two enzymes by the same compound may be attributable to differences in the active site milieu; a more open active site in factor Xa than in thrombin is consistent with a more solvated catalytic His57 in the former than in the latter. The upper limit of the rate constant pertains to the catalytically competent form of the enzyme in which all His57 is in the basic form. The value of k_{lim1} = 114 ± 5 $M^{-1}\ s^{-1}$ is 20 times smaller than k_{lim1} = 2366 ± 152 $M^{-1}\ s^{-1}$ in 0.15 M NaCl for thrombin.[20]

Reactivation of factor Xa from its PMN-inhibited adducts

Reactivation of factor Xa from the phosphonyl adducts in buffers containing 0.7 M NaCl in the pH range 6.5–11.1 failed to show the contribution of the enzyme in the reactivation process. The reactivation rates were not pH dependent at pH < 9 and increased dramatically at pH > 9. Addition of phospholipids, particularly PS, regardless of the length of the alkyl chain, improved the reactivation rates of factor Xa. Most probably a conformational change in factor Xa is triggered by the binding of PS which allows for proper positioning of His57 for catalysis.

The mechanism of reactivation of factor Xa

Reactivation of factor Xa from the phosphonyl adducts is due to the intramolecular displacement of the active-site Ser by the β-carbonyl group in the phenacyl ligand. Details of the mechanism of reactivation are outlined in Scheme 1. The general base-catalyzed hydration of the phenacyl group is followed by the intramolecular



Scheme 1.

nucleophilic attack by the anion of carbonyl hydrate at the phosphorus and subsequent release of Ser195. The observed rate constant for reactivation can be expressed in terms of elementary rate constants of the consecutive reaction scheme as in eq (8):

$$k_r = \frac{k'_h k_c}{k_{-h} + k_c} \tag{8}$$

In non-enzymic hydrolysis reactions of PMNs, hydration of the carbonyl, k_{-h}, is the rate-limiting step and cyclization, k_c, is faster than dehydration of the carbonyl, k_{-h}.[24] The value of $k_{-h} \sim 10^6$ s^{-1} calculated for nonenzymic hydrolysis of 4-CH_3-PMN, is not expected to be different for the enzyme-catalyzed reaction. Using the explicit values of the rate constants for the hydrolysis of 4-CH_3-PMN[24] and eqs (9) and (10), the rate constants for cyclization and enzyme-catalyzed hydration can be determined:

$$k'_h = k_{HOH}[HOH] + k_{HO}[HO^-] + k_B[B^-] + k_{enz} f_{His} \tag{9}$$

$$f_{His} = \frac{K_a}{K_a + H^+} \tag{10}$$

The rate constant k'_h represents the cumulative effect of water-, hydroxide ion-, buffer- and enzyme-catalyzed hydration. At pH 6.3 the fraction of deprotonated His is $<2\%$, thus the His general base-catalyzed hydration can be neglected. The value of k_r at pH 6.3 is indicated as k_{im2} in Table 3 and now the value for k_c can be determined from eq (11);

$$k_c = \frac{k_{-h} k_{lim\,2}}{k_{HOH}[HOH] + k_{HO}[HO^-] + k_B[B^-] - k_{lim\,2}} \tag{11}$$

The calculation yields $k_c = 3 \times 10^3$ s^{-1} for factor Xa. At the upper plateau of the pH profile for the reaction, the enzyme fully catalyzes hydration of the carbonyl, thus the enzyme-catalyzed contribution can be calculated using k_{lim1}, the upper limit of k_r;

$$k_{enz} = \frac{k_{lim\,1}(k_{-h} + k_c)}{k_c} \tag{12}$$

The calculation yields $k_{enz} = 5 \times 10^{-2}$ s^{-1} for factor Xa. Following the same sequence of calculations for thrombin,[20] yields $k_c = 7 \times 10^4$ s^{-1} and $k_{enz} = 9 \times 10^{-3}$ s^{-1}. Thus, formation of the oxyphosphorane intermediate is significantly slower than dehydration of the carbonyl hydrate, but even enzyme-catalyzed hydration is slower than subsequent steps.

Conclusions

The goal of this study was to test the inhibitory potency of the PMNs with factor Xa and the time scale and conditions under which factor Xa recovers from the phosphonyl adducts. Efficient inactivation on the minutes time scale at neutral pH and recovery times on the hours time scale have been found for the systems. Since reactivation is intramolecularly catalyzed, the recovery times observed are directly applicable to physiological conditions as shown for t-PA.[18] This is an asset for medical application.

Experimental

Materials

Human factor Xa, MW 46000 and activity 227.25 IU/mg in pH 7.4, 0.02 M Tris buffer, 0.7 M NaCl was purchased from Enzyme Research Laboratories, Inc. Factor Va, MW 168000 and activity 2190 U/mg in 50% glycerol and 2 mM $CaCl_2$ was purchased from Haematologic Technologies, Inc. (Essex Jct., VT). Solid *N*-Tosyl-Gly-Pro-Arg 7-AMC hydrochloride, >99% pure (TLC), was purchased from Sigma Chemical Co. 4-X-phenacyl 4-nitrophenyl methylphosphonate esters (X = H, CH_3, OCH_3) were prepared as reported in the literature.[25] The racemic mixtures were separated into the individual enantiomers by HPLC using a CHIRACEL OJ chiral column purchased from JT Baker following the method described earlier.[20] C6PS, the sodium salt of L-phosphatidylserine (PS) from brain and chloroform solution of L-phosphatidylcholine (PCh) from egg were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories.

Equipment

The release of 4-nitrophenol and the concentration of protein were measured using a Perkin–Elmer Lambda 6 or Lambda 7 UV–vis spectrophotometer connected to a PC. All fluorometric measurements were performed using an SLM-Aminco SPF-500C spectrofluorometer connected to a PC. LUV was prepared by using the Mini-Extruder purchased from Avanti Polar Lipids, Inc. The pH was measured using the Radiometer PHM82 electronic readout interfaced to a Fisher pH combination electrode. The temperature was monitored using a temperature probe connected to a digital readout device. Reaction mixtures and cuvettes were thermostated

using either a Neslab RTE-4 or a Lauda 20 circulating water bath. Most of the reactions were run in 1.5 mL polypropylene microcentrifuge tubes and the reaction mixtures were continuously stirred by placing a magnetic micro stirrbar inside the tubes. All buffers were filtered through a 0.22 μm pore Cameo nylon filter. Positive displacement Gilson and Rainin Microman pipettes with plastic tips were used for delivery of enzyme and substrate solutions. All data reductions using predefined and custom defined equations were performed using the software GraFit 3.0.[26]

Enzyme activity assay

Initial velocity measurements for factor Xa were done by using the fluorogenic substrate *N*-tosyl-Gly-Pro-Arg 7-AMC. The assay buffer at pH 8.0 contained 0.02 M HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 15% DMSO and 0.1% PEG-4000. The release of 7-AMC was recorded for 2 min in 1 s interval. When assaying samples containing phospholipids, the assay buffer included 10–20 mM EDTA and no $CaCl_2$.

Preparation of large unilamellar vesicles (LUV)[27,28]

A mixture of 25 mol% PS and 75 mol% PCh in chloroform was dried under a stream of dry N_2 in a glass vial. The required volume of the buffer of choice was added to obtain a final concentration of 2 mg phospholipid/mL and the mixture was vortexed. After 30 min, vesicles were produced by extrusion through 0.1 μm polycarbonate membranes by using the Mini-Extruder. The freeze–thaw method was repeated 3 times for generating uniformly sized vesicles. In order to accelerate thawing, a water bath at 60 °C was used. Flash-freezing in liquid nitrogen gave similar results to freezing in ethylene glycol at −40 to −50 °C.

Nitration of factor Xa

Nitration of factor Xa was performed by following the method of J. F. Riordan and B. L. Vallee.[29,30] One hundred microliters of 0.8 mg/mL stock solution of factor Xa was mixed with 20 μL pH 8.5, 0.1 M Tris buffer and 10 μL 35 mM tetranitromethane in ethanol. An excess of 200-fold tetranitromethane was used. Reaction occurred under continuous mixing for 40 min at room temperature. Nitrated factor Xa was purified by gel-filtration on Sephadex G-25 using pH 7.5 0.02 M HEPES buffer pH 7.5, 0.7 M NaCl as elution buffer. The eluted fractions were assayed for protein content using Bio-Rad protein assay reagent and for enzyme activity using *N*-tosyl-Gly-Pro-Arg 7-AMC. Preliminary separations were run with blue dextran and plasmin. A calibration curve was generated for the protein assay using known concentrations of factor Xa. Nitrated factor Xa was scanned in the region 300–500 nm.

Kinetics of inactivation of factor Xa by PMNs

Samples of 20 μL from the 21.3 μM stock solution of factor Xa were diluted ~50-fold with pH 5.7–8.0, 0.02 M MES/HEPES buffer, 0.15 M NaCl, and 0.1% PEG-4000, vortexed and divided in 50 μL portions into 1.5 mL microcentrifuge tubes, then equilibrated at 7.0 ± 0.1 °C. A ~11 mM stock solution of the inhibitor was prepared in dry acetonitrile and diluted before use with dry methanol. Inhibition was initiated by adding 8–10 μL of a diluted solution of PMNs at one of a series of concentrations to each microcentrifuge tube, but the reference sample. Mixing was done by a micro stirrbar placed inside the microcentrifuge tube. Samples of 25 μL were taken at 2 and 3 min from the reaction mixture and were assayed for amidolytic activity. Experiments were performed in duplicates for each reaction time and inhibitor concentration.

Reactivation of factor Xa from its adducts formed with (−)-PMNs

Fifty-five microliters of diluted factor Xa solution (4.8 μM) was mixed with 3–4 μL ~10 mM inhibitor stock solution and incubated for 25 min on ice. Reactivation started by adding the required buffer in the 7.8–11.0 pH range, containing 0.02 M Bis-Tris propane/CAPS, 0.7 M NaCl, and 0.1% PEG-4000, and was followed at 25.0 ± 0.1 °C. Samples of 10–25 μL were taken and assayed for amidolytic activity.

Reactivation of factor Xa from its adducts formed with (+)-PMNs

Inactivation of factor Xa by (+)-PMNs proved to be difficult to accomplish. Partial inhibition was achieved at pH 6.5 in 0.025 M citrate buffer.

Reactivation of factor Xa from its adducts formed with (−)-4-CH_3-PMN in the presence of C6PS

Ten microliters of C6PS in a 10 mg/mL chloroform solution was dried under a stream of dry N_2 in a glass vial. The solid obtained was dissolved in 600 μL Bis-Tris propane, Tris, or CAPS buffer in the range pH 6.6–10.3 and mixed well. Samples of inhibited factor Xa at approx. pH 6.5 were diluted with these reactivation buffers and reactivation was followed at 25.0 ± 0.1 °C by assaying for factor Xa amidolytic activity at given time intervals, 10–30 min.

Reactivation of factor Xa inhibited by (−)-PMNs and (+)-PMNs in the presence of LUV

Twenty-five microliters of diluted factor Xa solution (5.0 μM) was mixed with 3–5 μL inhibitor solution. After 90–120 min, 360 μL of the reactivation buffer was added, which contained 2 mg phospholipid/mL as LUV in 0.02 M Tris/Bis-Tris propane/CAPS, 0.15 M NaCl, and 0.1% PEG-4000. The reactivation mixture was thermostated at 25.0 ± 0.1 °C and continuously stirred. Samples were drawn at 10–30 min intervals and assayed for factor Xa activity. The assay buffer contained 10 mM EDTA.

Reactivation of factor Xa inhibited by (−)-PMNs and (+)-PMNs in the presence of LUV and factor Va

Due to the high cost of factor Va, experiments were run only for (−)-PMN, (+)-PMN, (−)-4-CH_3-PMN and

(+)-CH_3-PMN. The procedure was similar to the description above. The only difference was that factor Va was added in stoichiometric amount to form a 1:1 complex with factor Xa. The assay buffer contained 20 mM EDTA.

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