Dramatic enhancement of the catalytic activity of coagulation factor IXa by alcohols

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Abstract The coagulation factor IXa (FIXa) exhibits a very weak proteolytic activity towards natural or synthetic substrates. Upon complex formation with its cofactor FVIIIa and Ca^{2+}-mediated binding to phospholipid membranes, FIXa becomes a very potent activator of FX. The presence of FVIIIa has no effect on the cleavage of peptide substrates by FIXa, however. We found that several alcohols dramatically enhance the catalytic activity of human FIXa towards synthetic substrates. Substrates with the tripeptidyl moiety R–D-Xxx–Gly–Arg are especially susceptible to the enhanced FIXa catalysis. Maximal increase up to 20-fold has been measured in the presence of ethylene glycol. We suggest that alcohols modify the conformation of FIXa rendering the active-site cleft more easily accessible to tripeptide substrates with a hydrophobic residue in the P3-position.

Key words: Coagulation factor IXa; Synthetic substrate; Chromogenic assay

1. Introduction

The blood coagulation factor IXa is a trypsin-like serine proteinase circulating as a single-chain zymogen FIX in the plasma [1,2]. In the course of coagulation, FIX is activated and converted to its two-chain form FIXaβ by the TF–FVIIa complex in cooperation with FXa and FXIa [3,4]. On phospholipid surfaces FIXaβ assembles in a calcium-dependent fashion with factor VIIIa forming the so-called intrinsic Xase [5]. Its importance in the coagulation process is indicated by the bleeding disorders caused by mutations in either component: mutations in FVIII cause hemophilia A, whereas mutations in FIX give rise to hemophilia B [6].

FIXa possesses interesting enzymatic and structural properties. Compared to other coagulation enzymes, it exhibits only extremely weak proteolytic activity against natural or synthetic substrates in the absence of the cofactor FVIIIa [5]. However, complex formation with FVIIIa on the phospholipid surface leads to a potent activator of factor X [5,7], while the catalytic activity towards synthetic substrates is not enhanced [5]. The recently solved X-ray crystal structure of active-site inhibited full-length porcine FIXa shows an active-site geometry similar to that of other trypsin-like proteinases of high catalytic activity [8]. FIXa displays several characteristic differences, however.

Its essential role in the coagulation cascade suggests FIXa could be an important target for drug design and diagnostic efforts. The low catalytic activity and the lack of suitable synthetic substrates limit direct chromogenic or fluorogenic assays. Instead, FIXa is assayed under physiological conditions by activation of FX followed by chromogenic determination of resulting FXa activity. This indirect test is clearly disadvantageous in a variety of biotechnological fields, e.g. screening of inhibitors, which are well established for other coagulation enzymes.

In this report, we show that certain alcohols dramatically increase the catalytic activity of FIXa towards several synthetic substrates. This finding opens the way for direct FIXa assays in a variety of applications. The molecular aspects of this effect are discussed in terms of the X-ray crystal structure and the observed active site geometry of FIXa.

2. Materials and methods

2.1. Materials

Human FIXaβ, FXa, FXIa, FXIIa and plasma kallikrein were purchased from Kordia (Leiden, The Netherlands). Bovine thrombin was prepared according to Walsmann [9]. N-terminally truncated recombinant FIXa comprising the EGF-2 domain and the catalytic domain was prepared as described elsewhere [10]. The chromogenic peptide substrates MOC–D-Nle–Gly–Arg–pNA, MS–D-HHT–Gly–Arg–pNA, MS–D-CHG–Gly–Arg–pNA and Z–Glu–Gly–Arg–pNA were supplied by Pentapharm Ltd. (Basel, Switzerland); Bz–Ile–Glu–Gly–Arg–pNA (S-2222) was from Haemochrom Diagnostica GmbH (Essen, Germany). All reactions were performed in Tris buffer at 25°C (0.05 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, pH 7.4). The alcohols were from Sigma (Deisenhofen, Germany). The alcohols and all other chemicals used were of analytical grade.

2.2. Influence of alcohols on the catalytic activity of FIXa and of other enzymes

The measurements were carried out on a microplate reader (MR 5000, Dynatech, Denkendorf, Germany) at 25°C. The test medium consisted of 200 μl Tris buffer containing 0–60% of the respective alcohol, 25 μl of aqueous substrate solution (10 mM) and 20 μl of enzyme solution. The change in optical density was measured at 405 nm. The following concentrations were used for the different enzymes: FIXa (0.14 μM), rFIXa (0.48 μM), FXa (0.0016 μM), FXIa (0.0012 μM), FXIIa (0.0045 U/ml), plasma kallikrein (0.018 U/ml). The assays were performed in triplicate.

2.3. Determination of kinetic constants

Twenty-five microlitres of aqueous substrate solution and 20 μl of enzyme solution were added to 200 μl of Tris buffer (without or with 25% ethanol or 40% ethylene glycol, respectively). At least five concentrations of the substrate (between 0.25 and 4 mM) were used. The concentration of FIXa was 0.1 μM, and that of rFIXa was 0.48 μM. The measurements were carried out on a microplate reader at 25°C. Kinetic constants were determined from the initial rates of hydrolysis

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Abbreviations: FVIIIa, factor VIIIa; FIXa, factor IXa; rFIXa, recombinant factor IXa; FXa, factor Xa; FXIa, factor XIa; FXIIa, factor XIIa; MOC, methoxycarbonyl; MS, methylsulfonyl; HHT, hexahydrotyrosyl; CHG, cyclohexylglycyl; PEG, polyethylene glycol; TF, tissue factor

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using Lineweaver-Burk plots. A linear regression programme was used for calculation of the contants. The constants reported are means from at least three determinations.

3. Results

3.1. Enhancing effect of ethanol on the catalytic activity of FIXa

We screened several chromogenic tripeptide substrates for activity with human FIXa and found very low catalytic activity. As described by Castillo et al. [11], the regression lines in Lineweaver-Burk plots go approximately through the origin, allowing determination of the specificity constants k_{cat}/K_m only. For all the substrates tested, the substrate concentration was probably much lower than K_m. As substrate concentrations could not be increased due to their limited solubility, we used different additives to dissolve substrates at high concentrations. To our surprise we found that ethanol greatly enhances the catalytic activity of FIXa, especially for FXa substrates of the type R–D-Xxx–Gly–Arg–pNA. In contrast to the results obtained in the absence of alcohol, the Lineweaver-Burk plots yielded straight lines intersecting the y-axis near but not through the origin, indicating that the substrate concentrations were in the range of K_m. Using recombinant human FIXa (rFIXa), an identical enhancing effect was found with ethanol and other alcohols. Due to its availability and purity, rFIXa was used for further study of the effect of a panel of different alcohols and other cosolvents on catalytic activity. The substrate MS–D-HHT–Gly–Arg–pNA was used for most of the experiments.

3.2. Influence of alcohols on the catalytic activity of rFIXa

Among the monohydric alcohols, ethanol potentiated the activity of rFIXa nearly 6-fold and methanol nearly 4-fold (Fig. 1A). The maximal enhancing effect was found at an alcohol concentration (v/v) of 25%. Both *n*- and *i*-propanol as well as *t*-butanol up to a concentration of 10% likewise increased the catalytic activity, while higher concentrations lead to a drastic activity decrease compared to alcohol-free buffers. Some other alcohols, e.g. *n*-butanol, *n*-amyl alcohol and *n*-octanol, showed similar enhancing effects at low concentrations; they were, however, not miscible with buffers at concentrations higher than 10%.

The most striking effect on the catalytic activity of rFIXa was found for the dihydric alcohol ethylene glycol (Fig. 1B). The rate of cleavage of the substrate MS–D-HHT–Gly–Arg–pNA was enhanced more than 10-fold. In contrast to ethanol and methanol, which attained maximal effects at concentrations between 15 and 25%, the catalytic activity of rFIXa became maximal at ethylene glycol concentrations between 25 and 40%. Of the other dihydric alcohols, only 1,2-propanediol caused a noteworthy increase in enzyme activity, with a concentration dependence resembling that of ethylene glycol. Dihydric alcohols with a larger separation between the two hydroxyl groups such as 1,3-propanediol (Fig. 1B) and 1,4-butanediol (not shown here) affected the rFIXa activity only to a slight extent.

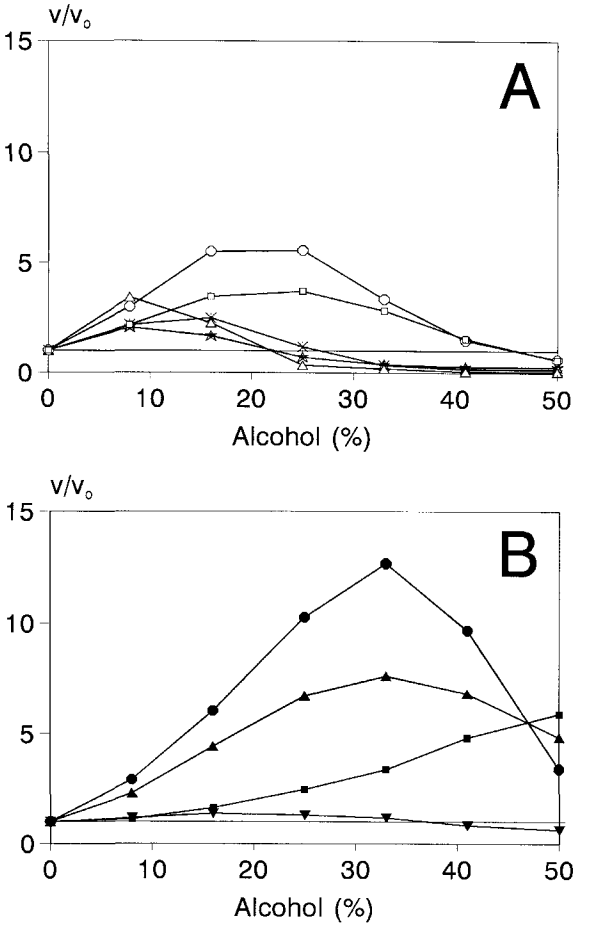

Fig. 1. Influence of monohydric (A) as well as dihydric alcohols and glycerol (B) on the cleavage of MS–D-HHT–Gly–Arg–pNA (1.02 mM) by rFIXa (0.48 μM) at pH 7.4. vo: Initial velocity without alcohol; v: initial velocity in the presence of alcohol; alcohols: methanol (□), ethanol (○), *n*-propanol (△), *i*-propanol (*), *t*-butanol (☆), ethylene glycol (●), 1,2-propanediol (▲), 1,3-propanediol (▼), glycerol (■).

Another concentration dependence was found for alcohols possessing even more hydroxyl groups. With glycerol, rFIXa activity increased continuously up to 6-fold at a 50% concentration (Fig. 1B). The same concentration dependence was found with *i*-erythritol (not shown here); the enhancing effect, however, amounted to 1.5-fold at maximum.

Table 1
Kinetic constants for cleaving FXa substrates by rFIXa at pH 7.4 in the absence and presence of 33% ethylene glycol (mean ± SD)

Substrate	Concentration of ethylene glycol				Amplification of k_{cat}/K_m
	0%	33%			
	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	
MOC–D-Nle–Gly–Arg–pNA	51.4 ± 6.8	2.01 ± 0.88	3.62 ± 1.59	558 ± 17	10.8
MS–D-CHG–Gly–Arg–pNA	86.9 ± 9.1	5.67 ± 1.37	1.43 ± 0.19	3920 ± 450	45.1
MS–D-HHT–Gly–Arg–pNA	82.3 ± 7.9	6.11 ± 1.50	5.99 ± 1.62	1028 ± 34	12.5

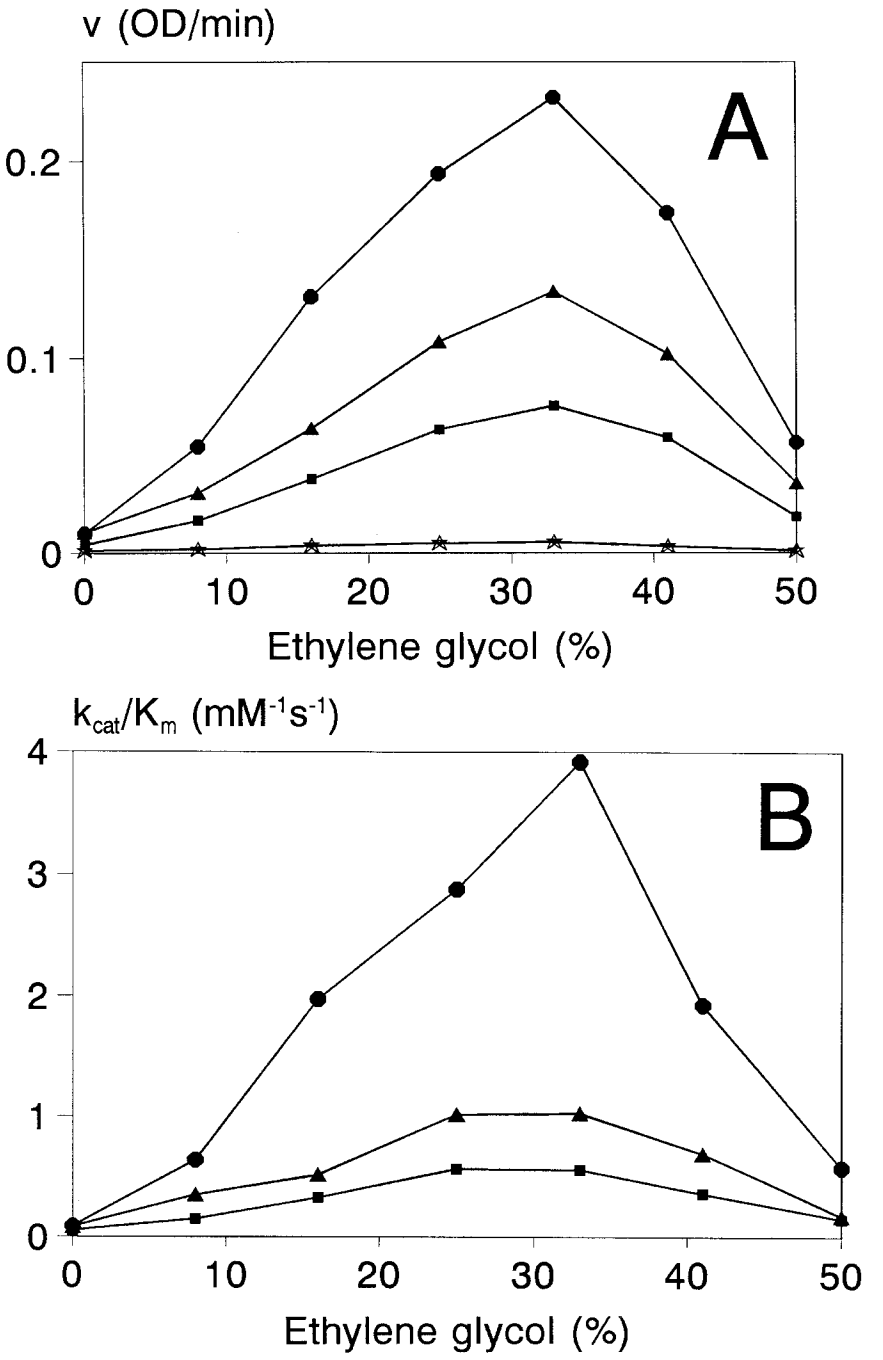

Fig. 2. Influence of ethylene glycol on the catalytic activity of rFIXa (0.48 μM) at pH 7.4. A: Initial velocity; B: specificity constants k_{cat}/K_m; substrates: MS–D-CHG–Gly–Arg–pNA (●), MS–D-HHT–Gly–Arg–pNA (▲), MOC–D-Nle–Gly–Arg–pNA (■), Bz–Ile–Glu–Gly–Arg–pNA (☆).

The potentiating effect of ethylene glycol and glycerol was abolished by derivatisation of the hydroxyl groups. Diethylene glycol, for example, caused only a 2-fold potentiation of rFIXa activity. The same hold for ethylene glycol monomethyl ether and for the glycerol derivative glycidol (2,3-epoxy-1-propanol) (not shown here). The polyhydric alcohol PEG inhibited substrate cleavage with increasing concentration.

3.3. *Influence of solvents on the catalytic activity of rFIXa*

Solvents lacking hydroxyl groups, such as dimethylformamide, dioxane, acetone and acetonitrile inactivated rFIXa in a similar manner as PEG. The same effect was seen with hydroxyacetone, but also with urea and guanidinium. Dimethylsulfoxide up to a concentration of 30% did not inhibit rFIXa's activity, but had only little potentiating effect (1.5-fold; not shown here).

3.4. *Effect of ethylene glycol on the cleavage of different substrates*

Using ethylene glycol, which showed the most remarkable enhancing effect on the catalytic activity of rFIXa (Fig. 1), the catalytic activity of rFIXa on different substrates was further

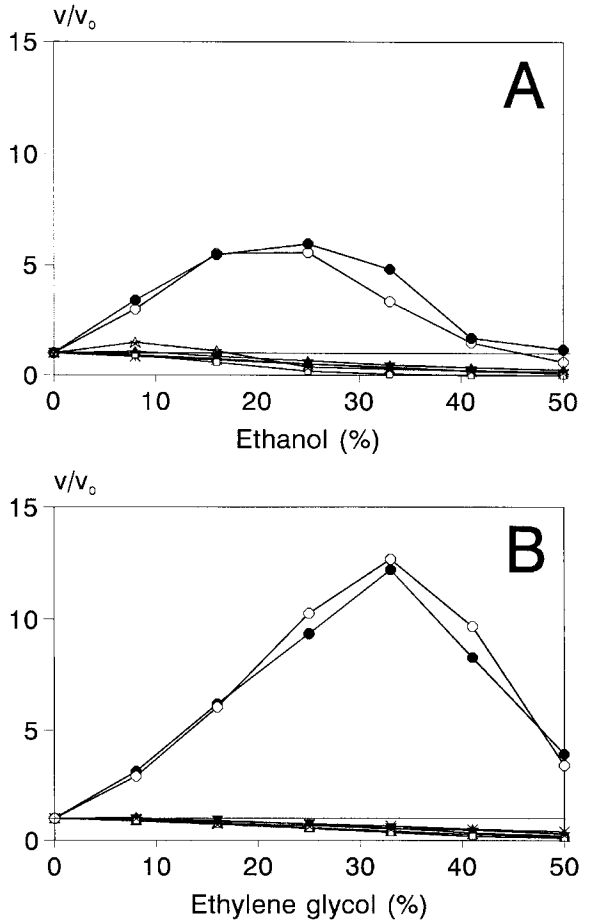

Fig. 3. Effect of ethanol (A) and ethylene glycol (B) on the cleavage of MS-D-HHT-Gly-Arg-pNA (1.02 mM) by native (●) and recombinant FIXa (○), plasma kallikrein (☆), FXIIa (□), FXIa (▲), FXa (▼) and thrombin (*) at pH 7.4.

investigated (Fig. 2A). The strongest enhancement was found for the substrate MS-D-CHG-Gly-Arg-pNA in the presence of 33% ethylene glycol, with the initial velocity of substrate cleavage potentiating up to more than 20-fold.

All substrates containing a D-amino acid in P3 displayed large enhancement effects in the presence of ethylene glycol. In contrast, substrates with an L-amino acid in P3 were hydrolyzed very slowly by FIXa, even in the presence of ethylene glycol or other alcohols. Fig. 2A displayed the cleavage rates for the selective FXa substrate Bz-Ile-Glu-Gly-Arg (S-2222). The velocities determined in the absence and presence of 33% ethylene glycol were very low (4.2 and 8.7 mOD/min, respectively). Similarly low velocities were found with the substrate Z-Glu-Gly-Arg-pNA (not shown), reported by Cho et al. [12] to be the best IXa substrate.

3.5. Determination of kinetic constants

The plots of initial velocity data versus substrate concentration were approximately linear for all substrates studied, indicating that the highest substrate concentrations never approached K_m. Correspondingly, the regression lines in Lineweaver-Burk plots ran through the origin disallowing resolution of individual k_{cat} and K_m parameters. These K_m values are probably above 10 mM. Values of k_{cat}/K_m were determined to be very low for the substrates MOC-D-Nle-Gly-Arg-pNA, MS-D-HHT-Gly-Arg-pNA and MS-D-CHG-Gly-Arg-pNA (Table 1).

At increasing concentrations of the effective alcohols, k_{cat}/K_m increased dramatically. The dependence on ethylene glycol concentration for three different substrates is shown in Fig. 2B. Maximal specificity constants were found for 33% ethylene glycol (Table 1). For the substrate MS-D-CHG-Gly-Arg-pNA, the specificity constant k_{cat}/K_m showed a 40-fold increase, while the amplification was 10-fold in case of MOC-D-Nle-Gly-Arg-pNA and MS-D-HHT-Gly-Arg-pNA. Due to this enhanced activity in the presence of ethylene glycol, individual K_m values could also be determined amounting to 1–10 mM (Table 1).

3.6. Influence of ethylene glycol on other clotting enzymes

Native and recombinant human FIXa were identical in exhibiting this dramatic alcohol enhancement (Fig. 3). In stark contrast, the catalytic activity of other trypsin-like enzymes was decreased by alcohols. Fig. 3A,B show the effect of ethanol and ethylene glycol on the activity of other members of the intrinsic coagulation pathway, namely plasma kallikrein and factors Xa, XIa, XIIa and thrombin. Only plasma kallikrein tolerated alcohols up to a concentration of 10–15%, while all the other enzymes studied were inhibited.

4. Discussion

Fig. 4 shows the catalytic domain of porcine FIXa around the active site, with subsites S1 to S3/S4 occupied by the D-Phe-Pro-Arg moiety of the covalently bound PPACK inhibitor [8]. Compared to FXa [13], the 99 loop of FIXa (chymotrypsinogen numbering) contains two additional residues. In FIXa, the phenolic side chain of Tyr^{99} does not extend into the S2 subsite restricting it as in FXa, but is displaced to occupy the aromatic S3/S4 subsite. Thus, the medium sized proline ring of PPACK can be accommodated at the S2 subsite, while the benzyl side chain of its D-Phe residue cannot slot into the S3/S4 subsite, presumably giving rise to a non-optimal overall binding of the inhibitor. The Arg side chain of PPACK is inserted into the S1 pocket of FIXa in the usual manner, i.e. with its distal guanidino group hydrogen bond/salt bridge connected with the Asp^{189} carboxylate and Glu^{219} O. In the crystal structure of FIXa, this latter carbonyl is only weakly defined by electron density, suggesting some oscillation caused by a tensed conformation energetically favourable only for Gly residues. Glu^{219} is strictly conserved in all FIX species sequenced so far, but is replaced by a glycine in all other serine proteinases with a trypsin-like S1 pocket. In free FIXa, this S1 pocket is assumed to be partially closed (cryptic), what would be in good agreement with the extremely low affinity of benzamidine and related S1 accommodating inhibitors for FIXa [8].

The geometries of both the S3/S4 cleft and the S1 pocket might be modified externally, e.g. by certain alcohols in an as yet unknown manner. A possibility is that interaction with alcohols restores deformed or structurally flexible patches in the active site of FIXa. Alternatively, specific alcohols could influence surface conformations in the vicinity of Tyr^{99}, most likely the loop comprising residues 94–99; this could lead to a removal of the unfavorable interactions mentioned before for D-configurated P3 residues. This would allow a more precise

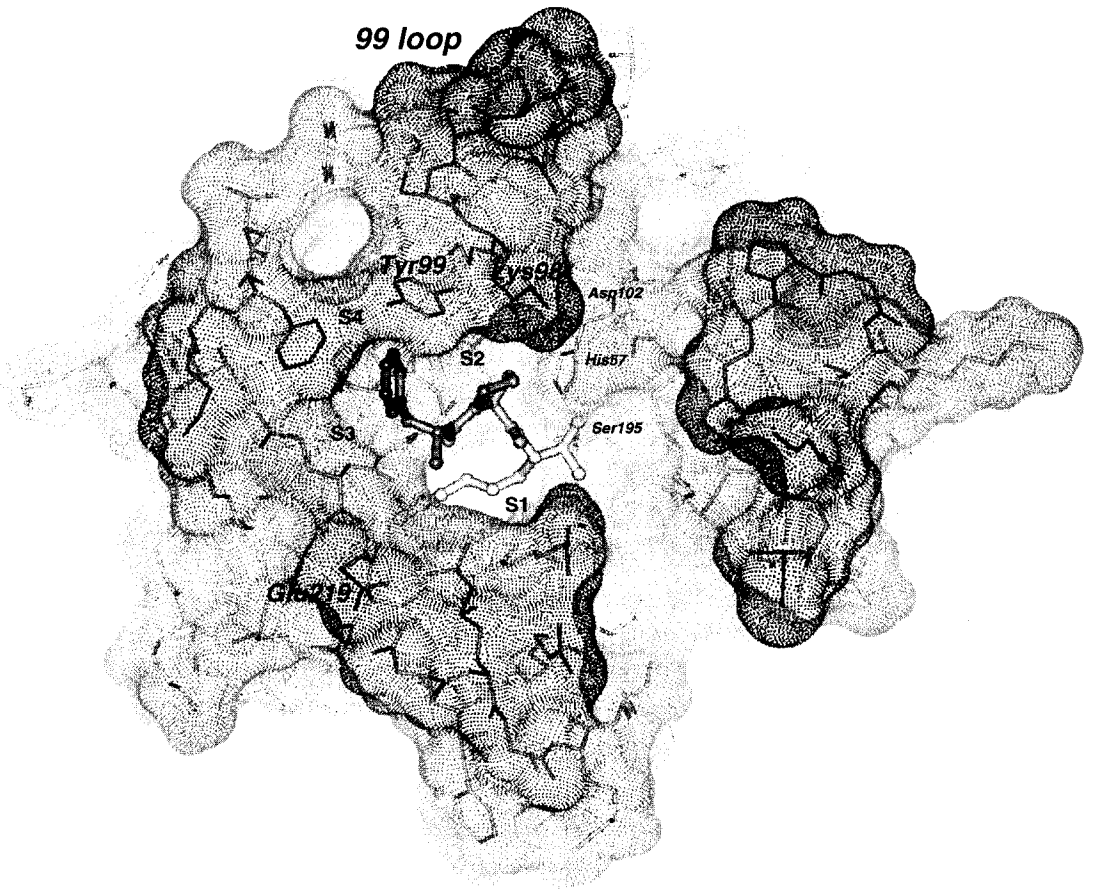

Fig. 4. PPACK bound to the active site of FIXa (displayed as stick model) superimposed with a Connolly dot surface; according to [8]. The S1-specificity pocket (central hole) opens to the left of the catalytic triad residues Ser^{195}, His^{57} and Asp^{102}. The P1-Arg side chain of PPACK extends into this S1-pocket forming a salt bridge to Asp^{189} (not shown) and a hydrogen bond to the carbonyl oxygen of Glu^{219}. P2-Pro and P3–D-Phe of PPACK contact the side chains of Lys^{98} and Tyr^{99}, which are part of the 99 loop.

binding at the active site, with beneficial effects on the catalytic efficiency. This hypothesis seems to be corroborated somewhat by the much larger catalytic activity enhancement for substrates containing a D-amino acid compared to substrates containing an L-amino acid at P3. For tripeptidyl substrates that bind with an extended conformation, only side chains of D-configured P3 residues will interact with S3/S4 and might thus collide with Tyr^{99}. Further support of this hypothesis comes from mutational analyses [10]; transplantation of the 99 loop of FXa to FIXa increases the catalytic activity towards k_{cat} and K_m values similar to those observed for FIXa in the presence of ethylene glycol.

It is unlikely that this alcohol effect is brought about by a change in the dielectric constant of the solvent. Rather, the differential effects of the various alcohols point to interactions at a distinct binding site. Both the number and the position of hydroxyl groups as well as the number of separating methylene groups is of paramount importance. The amplification by monohydric alcohols increases in the order *t*-butanol < *i*-propanol < *n*-propanol < methanol < ethanol. Thus, a hydroxymethyl group with one adjacent methyl group seems optimal. In the case of two hydroxyl groups, one adjacent methyl group is tolerated. The ranking order of the most effective alcohols is ethanol < 1,2-propanediol < ethylene glycol. The effect observed with 1,3-propanediol is only 10–15% of that seen with ethylene glycol or 1,2-propanediol, respectively; thus, intervening methylene groups have detrimental effects on the effectiveness of alcohols.

An important result of this work is that FIXa shows increased activity in the presence of certain alcohols. This finding opens the possibility for simple direct chromogenic or fluorogenic assays for determination of active FIXa; it facilitates many applications such as screening for specific inhibitors directed against FIXa as potential antithrombotic drugs, or routine diagnostics of FIX and FIXa in plasma or plasma products. The fact that other coagulation enzymes, especially the more abundant FXa and thrombin, are inhibited by alcohols is of great importance, since side reactions due to the presence of these enzymes would be depressed.

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