Converting blood coagulation factor IXa into factor Xa: dramatic increase in amidolytic activity identifies important active site determinants

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The coagulation factors IXa (fIXa) and Xa (fXa) share extensive structural and functional homology; both cleave natural substrates effectively only with a cofactor at a phospholipid surface. However, the amidolytic activity of fIXa is 10⁴-fold lower than that of fXa. To identify determinants of this poor reactivity, we expressed variants of truncated fIXa (rf9a) and fXa (rf10a) in *Escherichia coli*. The crystal structures of fIXa and fXa revealed four characteristic active site components which were subsequently exchanged between rf9a and rf10a. Exchanging Glu219 by Gly or exchanging the 148 loop did not increase activity of rf9a, whereas corresponding mutations abolished reactivity of rf10a. Exchanging Ile213 by Val only moderately increased reactivity of rf9a. Exchanging the 99 loop, however, dramatically increased reactivity. Furthermore, combining all four mutations essentially introduced fXa properties into rf9a: the amidolytic activity was increased 130-fold with fXa substrate selectivity. The results suggest a 2-fold origin of fIXa's poor reactivity. A narrowed S3/S4 subsite disfavours interaction with substrate P3/P4 residues, while a distorted S1 subsite disfavours effective cleavage of the scissile bond. Both defects could be repaired by introducing fXa residues. Such engineered coagulation enzymes will be useful in diagnostics and in the development of therapeutics.

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Introduction

Coagulation factor IX (fIX) is a 415-residue zymogen circulating in the plasma (DiScipio *et al.*, 1977; Davie *et al.*, 1991). fIX is activated to fIXa through the cleavage of two bonds by either factor VIIa (fVIIa) or factor XIa (Fujikawa *et al.*, 1974; Lindquist *et al.*, 1978), releasing a 35-residue activation peptide (Kurachi and Davie, 1982; Lawson and Mann, 1991). fIXa assembles with factor VIIIa (fVIIIa) on the surface of endothelial cells or

activated platelets (van Dieijen *et al.*, 1981), forming the intrinsic Xase, a potent activator of factor X (fX). The importance of fIXa in haemostasis is indicated by the frequency of haemophilia B (Roberts, 1993) which afflicts 1 in 30 000 males.

fIX is a multi-domain protein. An N-terminal γ -carboxy glutamic acid (GLA) domain is followed by two epidermal growth factor-like (EGF) repeats, the activation peptide (AP) and a C-terminal serine protease domain with a trypsin-like active site (DiScipio *et al.*, 1978). This domain structure defines the fIX gene family of clotting factors (Furie and Furie, 1988), including also fVII, fX and protein C (PC). Within this family, fIXa has unique proteolytic properties. Complex formation of fIXa with fVIIIa on a phospholipid surface increases reactivity against the natural substrate fX 10⁶-fold (Duffy and Lollar, 1992), while virtually no cleavage of peptides with corresponding fX sequences was observed (McRae *et al.*, 1981; Castillo *et al.*, 1983). Further, sensitive synthetic substrates and specific low-molecular weight inhibitors of fIXa to assist the development of fIXa anticoagulants have not been found.

The remarkably different properties of fIXa and fXa—fIXa's most closely related homologue—have not been explained. Like fIXa, cleavage of fXa's primary physiological substrate (prothrombin) depends on a cofactor and a phospholipid surface (Jackson and Nemerson, 1980). However, fXa shows very high catalytic activity and specificity against synthetic substrates, especially prothrombin activation sequence analogues (Lottenberg *et al.*, 1981). Indeed, the specificity constant for preferred synthetic substrates differs by three to four orders of magnitude between fIXa and fXa, although both enzymes displayed a quite similar substrate cleavage profile (Cho *et al.*, 1984).

The X-ray structures of PPACK-inhibited porcine fIXa (Brandstetter *et al.*, 1995) and human fXa (Padmanabhan *et al.*, 1993; Brandstetter *et al.*, 1996) showed very similar active site structures. Several structural features, however, may account for the poor catalysis of fIXa (Figure 1). Glu219 (chymotrypsinogen numbering is used except when indicated otherwise) at the entrance frame of the S1 pocket is strictly conserved as a Glu in fIXa, but is found exclusively as a glycine in other trypsin-like proteases (Figure 2). Since the crystal structure of fIXa showed a backbone conformation allowed only for glycine residues, it was proposed that inhibitor binding induces the observed conformation of an otherwise more collapsed S1 pocket (Brandstetter *et al.*, 1995). Lys148, which is conserved in both enzymes, forms a salt bridge with Glu219 in fIXa, but points into the solvent in fXa. Tyr99 is part of a loop which defines the S2 pocket in fXa (Padmanabhan *et al.*, 1993). It presumably accounts for fXa's preference for a glycine at P2. Although conserved in both molecules,

Fig. 1. Stereo plot of the active site of porcine fIXa (thick grey and colour-coded sticks) superimposed with fXa (thin blue sticks). Disulfide bonds are coloured yellow. The inhibitor PPACK (magenta) is covalently bound to the catalytic Ser195 and His57 (orange) of fIXa. P1-Arg, P2-Pro and P3-D-Phe occupy the S1, S2 and S3/S4 subsites of fIXa, respectively. The S1 pockets of fIXa and fXa are virtually identical; notably differing residues occur elsewhere (red). Residue Tyr99 partially occupies the S3/S4 subsite of fIXa to block the P3-D-Phe of PPACK. The unique fIX residue Glu219 forms a salt bridge to Arg148 (human fIXa and rf9a have a Lys at this position) which, unlike the solvent-exposed orientation of Lys148 in fXa, points toward the S1 pocket. The strictly conserved Ile213 of fIXa—valine in most other trypsin-like serine proteases—is at the entrance of the S1 pocket near the catalytic Ser195.

Tyr99 is rotated by 90° in the fIXa structure which partially blocks the S3/4 subsite as compared with other trypsin-like proteases. Finally, Ile213, located close to the Ser195, is strictly conserved in fIX but found as a valine in most other trypsin-like serine proteases, including fX, fVII, PC, prothrombin and trypsin.

Using site-directed mutagenesis, we swapped residues or loops between recombinant des-GLA, des-EGF1 variants of fIXa and fXa (denoted rf9a and rf10a) to identify important active site determinants of the catalytic properties of rf9a and rf10a. In particular, we exchanged residue 219, residue 213 and residues around Tyr99 and Lys148. The GLA domain and first EGF domain were apparently flexible in the crystal structures of fXa (Padmanabhan *et al.*, 1993), fIXa (Brandstetter *et al.*, 1995) and also activated PC (Mather *et al.*, 1996); truncation of these domains is expected to enhance crystallizability. Since modifications of amidolytic activity are linked principally to the catalytic domain, the N-terminal truncations are not expected to introduce artefacts. Such variants, which have been used previously (Rezaie *et al.*, 1993; Rezaie, 1996), enabled us to use *Escherichia coli* for expression (*E. coli* is incapable of introducing post-translational modifications located in the GLA domain and EGF1 domain of full-length fIX and fX). Since heterologous cytoplasmic expression of disulfide bridge-containing proteins in general leads to the formation of insoluble protein aggregates (inclusion bodies), we developed an *in vitro* folding protocol to obtain active protein. These variants of fIXa and fXa are useful not only in structure–function studies, but should also facilitate inhibitor screening and structure-based inhibitor development.

Results

Expression, folding and purification of recombinant proteins

From 1 litre of *E. coli* cell culture, 50–100 mg of recombinant inclusion body material was solubilized and diluted into folding buffer. The folding mixture was subjected to anion exchange chromatography and correctly folded protein eluted in a single peak (Figure 3). After affinity chromatography on heparin–Sepharose, the zymogens were activated by incubation with Russell's viper venom (RVV-X) (Furie and Furie, 1976) which cleaved at Rn145–An146 (n refers to the natural protein numbering) and Rn180–Vn181 of rf9 and at Rn142–Sn143 and Rn194–In195 of rf10 as judged by SDS–PAGE and confirmed by electrospray mass spectrometry (ESMS). The masses were found to be 32 982 Da and 33 620 Da for rf9a and rf10a, respectively, consistent with the calculated masses of 32 973 Da and 33 612 Da. The final yield of homogenous purified active enzymes was ~5 mg/l cell culture. The domain structures of the recombinant proteins are shown in Figure 4. To establish the correct folding and formation of amidolytic activity, structural and functional properties of refolded proteins were compared with those of plasma-derived and tissue culture-expressed proteins. Comparison of amidolytic and inhibitor-binding properties with confirmed native amidolytic properties are shown in Table I. A crystal structure of rf10a showed the fXa fold (Padmanabhan *et al.*, 1993) and correct formation of disulfide bonds (unpublished data). Since, in addition, a tissue culture-expressed Glu219Gly mutant of fIXa had essentially the same amidolytic properties as the refolded Glu219Gly mutant of rf9a (A. Wolberg and D. Stafford,

Fig. 2. Sequence alignment of the catalytic domain of fIXa with several homologous coagulation enzymes. Conserved residues are boxed and shaded in yellow. Residues identified as characteristic active site determinants by comparison of the crystal structures of fIXa and fXa (Figure 1) are indicated in red. The segments exchanged between rf9a and rf10a to produce the rf9a/rf10a hybrids are coloured green. I213 and E219 are uniquely conserved in all nine known fIXa sequences but found as valine or glycine in most other trypsin-like serine proteases. Y99 of fIXa occurs in the 99 loop with an among fIXa species conserved two-residue insertion relative to fXa. K148 forms part of a loop whose length but not character is conserved between fIXa and fXa. The upper numbering is according to the chymotrypsinogen system; the lower numbering according to the fIXa system.

personal communication), these results confirm correct folding and the suitability of N-terminal truncation.

Catalytic activity of mutant enzymes

The fIXa-fXa hybrid mutants, shown in a sequence alignment with fIXa, fXa and related enzymes (Figure 2), were characterized by two approaches. Changes in catalytic properties were probed with the fXa substrate MOC-D-Nle-Gly-Arg-pNA. To localize effects associated with the primary specificity pocket, we measured the dissociation constant of *p*-aminobenzamidine (PABA), an S1-pocket-directed reversible inhibitor of serine proteases with trypsin-like specificity (Mares-Guia and Shaw, 1965). The observed kinetic parameters are listed in Table II.

The catalytic efficiency (k_{cat}/K_m) of rf10a was 10^4 -fold that of rf9a, and individual mutations significantly altered these efficiencies. Replacing six residues in the 99 loop of rf9a by four equivalent residues of rf10a [rf9a-NAAINK95_98TKET is denoted rf9a-99loop(f10)] resulted in a 20-fold increase in k_{cat}/K_m , while the converse mutation in rf10a [rf10a-TKET95_98NAAINK is denoted rf10a-99loop(f9)] reduced the specificity constant by a factor of 40, principally by increasing the K_m . A corresponding decrease in K_m probably explains the rf9a-99loop(f10) catalytic efficiency: for substrate concentrations used, K_m could only be estimated as >40 mM (no substrate saturation of steady-state velocities was observed for limiting substrate concentrations up to 10 mM which

should be detectable assuming $K_m = 40$ mM and an experimental uncertainty of 20%) for rf9a but could be determined as 5.1 mM for rf9a-99loop(f10). These results indicate an important role of the 99 loop in substrate binding rather than in catalytic turnover. Furthermore, this role is limited to sites P2–P4, since replacement of the 99 loop caused only slightly modified binding affinities for PABA, compared with its effect on K_m values.

Replacing residue 213 only moderately changed the amidolytic properties of both enzymes, predominantly by increasing the k_{cat} of rf9a-I213V and decreasing the k_{cat} of rf10a-V213I.

The conservation of Gly219 in all trypsin-like serine proteases (except fIX) is highlighted by the dramatically lowered catalytic efficiency of rf10a-G219E, with only 0.1% of the activity of rf10a. Since PABA binding was also decreased by more than two orders of magnitude, Gly219Glu clearly presented an altered S1 site geometry. Surprisingly, mutant rf9a-Glu219Gly showed neither a corresponding increase in catalytic efficiency over rf9a nor improved affinity for PABA. Glu219 thus is not the sole determinant of impaired binding and activity in fIXa, not even when considering only S1 interactions. Indeed, changing three residues in the 148 loop exerted a similar influence on the geometry of the S1 pocket: the mutations of rf9a-VFH145_147THE [hereafter denoted rf9a-148loop(f10)] and rf10a-THE145_147VFH [denoted rf10a-148loop(f9)] led to kinetic parameters similar to those of rf9a-E219G and rf10a-G219E, respectively.

Fig. 3. Typical anion exchange HPLC elution profile after folding. Correctly folded material elutes as a single peak at the beginning of the linear gradient (0.15–0.4 M NaCl), is identified by activity measurements (open columns; in relative units) and is essentially homogeneous (SDS–PAGE, inset). Comparison of recombinant protein with des–GLA plasma-derived fXa (gdfXa) confirms proteolytic processing to heavy (CD) and light chains (EGF).

Fig. 4. Domain structure (boxes) and disulfide bonds of rf9 and rf10 compared with plasma-derived fIX and fX. fIX and fX comprise the GLA domain, two EGF-like repeats and the catalytic domain, as well as the aromatic stack (AS) and the activation peptide (AP). rf9 and rf10 are des–GLA, des–EGF1 variants of the full-length native proteins. The arrows indicate activation sites of the zymogens. fX circulates in the blood in a two-chain form linked by a disulfide bridge. fIX circulates as a single-chain molecule.

If binding effects of mutations are independent, the free energies of binding of combinations of mutations are additive. In general, however, mutations will be coupled, leading to non-additivity as represented by a coupling free energy (Carter *et al.*, 1984; Wells, 1990; LiCata and Ackers, 1995). The proximity of residue 219 and the 148 loop (Figure 1) and the similar effects of corresponding mutants on catalysis and binding suggest such a structural and functional coupling. To examine such coupling, we constructed three multiple mutants. Combination of Gly219 and 148loop(f10) in rf9a led to partial restitution of wild-type properties in the double mutant as compared with the single mutants. The triple mutant rf9a-148loop(f10)-I213V-E219G possessed a catalytic effi-

ency more than twice that of rf9a. The quadruple mutant rf9a-99loop(f10)-148loop(f10)-I213V-E219G, however, displayed a catalytic efficiency 130-fold that of wild-type rf9a. In fact, its specificity constant was now closer to rf10a than to rf9a. Thus, coupling effects dominate substrate specificity.

Transition in substrate selectivity

The quadruple active site mutations effectively changed the catalytic efficiency of rf9a to that of rf10a. To characterize the mutants as rf9a-like or rf10a-like not only with respect to reactivity, we also examined substrate selectivity (defined here as the ratio of specificity constants). Since both wild-type enzymes preferentially cleave

substrates of the form X-Gly-Arg-pNA (Cho *et al.*, 1984), we varied the P3 residue to probe selectivity. Using the two substrates MOC-D-Nle-Gly-Arg-pNA and MS-D-Phe-Gly-Arg-pNA with rf10a and rf9a selectivity, respectively, we could identify a selectivity switch between rf9a and rf10a (Table III). The graduated selectivity between fIXa

Table I. Comparison of amidolytic (MOC-D-Nle-Gly-Arg-pNA) and inhibitor binding (PABA) properties of plasma derived and recombinant proteins^a

	MOC-D-Nle-Gly-Arg-pNA			PABA K_i (μ M)5f
	k_{cat} (s^{-1})	K_m (μ M)	k_{cat}/K_m ($s^{-1} M^{-1}$)	
Human fIXa ^b	n.d. ^c	n.d.	93	200
rf9a ^b	n.d.	n.d.	91	190
Human fXa	174	195	892 000	62
rf10a	182	186	983 000	60

^aData represent the mean of three independent measurements. The standard error was 5–10%.

^bDr Stürzebecher, personal communication.

^c K_m and k_{cat} could not be determined due to substrate solubility and high K_m values of fIXa and rf9a.

and fXa demonstrates the simultaneous roles of the structural determinants both in catalytic efficiency and also molecular recognition. As expected from direct contacts between the Tyr99 side chain and the P3-D-Phe of PPack (Figure 1), the 99 loop exhibited the largest impact on selectivity. Mutations more distant from the S3/S4 subsite modified selectivity only moderately, while combinations of mutations cooperatively changed selectivity ultimately to transform rf9a to an enzyme more like rf10a.

Discussion

We have examined important active site determinants of fIXa by exchanging them between recombinant variants of fIX and its closest homologue fXa (Figure 5) with two principal results. First, building the rf10a 99 loop into rf9a greatly increased catalytic efficiency, while the converse introduction of the rf9a 99 loop into rf10a comparably decreased catalytic efficiency; the altered K_m values showed an effective transplantation of substrate recognition properties between the two enzymes. Second, the structural determinants act cooperatively. Individual mutations introducing rf10a-residues at positions 219, 213

Table II. Kinetic constants for hydrolysis of MOC-D-Nle-Gly-Arg-pNA and binding constants for PABA^a

	MOC-D-Nle-Gly-Arg-pNA			PABA K_i (μ M)
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} M^{-1}$)	
rf9a	n.d. ^b	n.d.	93	200
rf9a-99loop(f10)	9.9	5.1	1970	120
rf9a-148loop(f10)	n.d.	n.d.	32	330
rf9a-I213V	n.d.	n.d.	123	370
rf9a-E219G	n.d.	n.d.	51	900
rf9a-148loop(f10)-E219G	n.d.	n.d.	77	310
rf9a-148loop(f10)-I213V-E219G	n.d.	n.d.	221	540
rf9a-99loop(f10)-148loop(f10)-I213V-E219G	107	8.8	12 100	170
rf10a	182	0.186	983 000	60
rf10a-99loop(f9)	107	6.0	17 800	360
rf10a-148loop(f9)	8.6	6.0	1400	3900
rf10a-V213I	120	0.198	605 000	58
rf10a-G219E	6.5	6.8	960	8200

^aData represent the mean of three independent measurements. The standard error was 5–20%.

^bSee footnote (c) in Table I.

Table III. Specificity constants (k_{cat}/K_m) for hydrolysis of MOC-D-Nle-Pro-Arg-pNA and MS-D-Phe-Pro-Arg-pNA^a

	k_{cat}/K_m ($s^{-1} M^{-1}$)		Selectivity D-Nle/D-Phe
	MOC-D-Nle-Gly-Arg-pNA	MS-D-Phe-Gly-Arg-pNA	
rf9a	93	118	0.8
rf9a-99loop(f10)	1970	1600	1.2
rf9a-148loop(f10)	32	37	0.9
rf9a-I213V	123	154	0.8
rf9a-E219G	51	69	0.7
rf9a-148loop(f10)-E219G	77	70	1.1
rf9a-148loop(f10)-I213V-E219G	221	179	1.2
rf9a-99loop(f10)-148loop(f10)-I213V-E219G	12 100	6700	1.8
rf10a	983 000	390 000	2.5
rf10a-99loop(f9)	17 800	10 200	1.7
rf10a-148loop(f9)	1400	900	1.6
rf10a-V213I	605 000	230 000	2.6
rf10a-G219E	960	510	1.9

The selectivity is defined by the ratio of the two specificity constants. Data represent the mean of three independent measurements. The standard error was 5–20%.

Fig. 5. Stereo plot showing residues and loops mutated in the active site fIXa (the colour code of Figure 1 is used). Residues and loops exchanged by site-directed mutagenesis are shown in green. The blue residues denote positions identical in the amino acid sequence of human fIXa and fXa. The 99 loop forms a large part of the S2 and S3/S4 subsites, thus determining substrate recognition and binding in accordance with its significant effect on the K_m values. The mutations at position 213 and 219 as well as the 148 loop are embedded in a conserved region (blue residues) located around the primary specificity pocket. Their close proximity illustrates coupling effects leading to a large increase in catalytic efficiency only upon combination of mutations.

and the 148 loop in rf9a had little effect on the catalytic efficiency of rf9a, while corresponding replacements in rf10a were mostly detrimental. However, combinations of these S1-pocket-located mutations acted cooperatively such that a quadruple mutant of rf9a had an increased amidolytic activity and substrate selectivity closer to rf10a than to rf9a.

Replacement of six residues located in the 99 loop of fIXa (Figure 5) by four residues of the equivalent loop of fXa exchanged both amidolytic reactivity and substrate selectivity between rf9a and rf10a. The different lengths of 99 loops of fIXa and fXa (Figure 2) generate divergent backbone conformations and probably the differing orientations observed for Tyr99 (Figure 1). In fXa, Tyr99 points towards His57, defining one side of a restricted S2 subsite, appropriate for the Gly preference for P2 of fXa substrates. In fIXa, however, Tyr99 is rotated toward the S3/S4 subsite (Figure 1) (Brandstetter *et al.*, 1995). This expands the S2 site and blocks the S3/S4 site relative to fXa, probably accounting for the inferior substrate affinities of rf9a. Such changes in the orientation of Tyr99 probably cause the large changes of synthetic substrate K_m values upon replacement of the 99 loop. They are probably also responsible for rf9a's and rf10a's different preference for D-Phe and D-Nle in P3, respectively. The identification of this structural switch thus is an important clarification of the role of Tyr99 in determining enzyme specificity. It provides structural arguments for an understanding of experiments such as that of Rezaie (1996), who demonstrated that interchanging residue 99 between fXa (Tyr) and activated PC (Thr) not only switched selectivity for chromogenic substrates but additionally altered interactions with inhibitors and macromolecular substrates.

The individual S1 pocket mutations Ile213Val, Glu219Gly and 148loop(f10) in rf9a did not improve the impaired catalytic efficiency and PABA-binding properties. However, both the replacement of the 148 loop and the replacement of Gly219 by Glu abolished reactivity of rf10a by disrupting the S1 pocket. Apparently, the backbone conformation at 219 suitable for catalysis is strained for non-glycine residues and can be formed only with additional stabilizing interactions, such as in the case of fIXa, by an apparent salt bridge interaction with Lys148 (Figure 1). This explains the cooperativity of the mutations and the necessity of combined mutations 219G, 213V and 148loop(f10) to increase significantly the catalytic efficiency of rf9a. Similar observations were made during attempts to engineer chymotrypsin-like specificity into trypsin (Perona and Craik, 1995; Hedstrom, 1996). Here, maintaining catalytic efficiency while altering specificity required the simultaneous replacement of several loops near the S1 pocket (Hedstrom *et al.*, 1992); single mutations in the S1 pocket reduced both specificity and reactivity. Combinations of mutations increased catalytic efficiency mainly through an increase in k_{cat} (Hedstrom *et al.*, 1994).

Similarly in our work, variant rf9a-99loop(f10)-148loop(f10)-I213V-E219G also showed a greatly increased k_{cat} , compared with rf9a-99loop(f10), while neither the K_m nor the K_i were improved. This suggests that the binding strength of the substrate to the enzyme is unchanged, but rather cooperativity of S1-pocket-directed mutations stabilizes the bound transition state. The stabilizing transition state binding geometry is more readily disrupted than the substrate binding geometries. This interpretation is supported further by the PABA K_i values

of the mutants, which are not correlated with the corresponding amidolytic activities. For example, the wild-type rf9a and the quadruple mutant have comparable K_i values, yet differ by more than two orders of magnitude in their catalytic activity. Apparently, the S1 pocket mutations either disturb the geometric relationship of the transition state stabilizing residues with each other, or alter the binding geometry (shape or orientation) of the P1 residue and in turn that of the transition state. Thus, although the P1 residue binds with a comparable affinity to both enzymes (estimated by the K_i of PABA), the orientation of the carbonyl group of the scissile bond to the catalytic serine and the residues of the oxyanion hole is probably altered. This is consistent with the observation of conformational variations of the S1 pocket to different binding modes of different inhibitors (Engh *et al.*, 1996) and also with results from the chymotrypsin–trypsin hybrid mutants described above (Hedstrom *et al.*, 1994), where differences in catalytic activity were found to be due mainly to differences in transition state stabilization.

Taken together, these results suggest a 2-fold origin of the low catalytic activity of fIXa. Firstly, several residues near the S1 pocket disfavour high-affinity binding of the transition state, predominantly by an unfavourable orientation of the scissile bond and the catalytic residues. Consequently, the k_{cat} of fIXa for cleavage of peptide substrates is much lower than for other clotting factors. Secondly, the S3/S4 subsite is restricted because of the location of the Tyr99 side chain triggered by the 99 loop. It sterically hinders high-affinity binding of P3 and P4 substrate residues. Consequently, the K_m (more specifically K_d) for favourable chromogenic substrates is found to be at least two to three orders of magnitude higher than for homologous proteases such as fXa, thrombin and trypsin (Lottenberg *et al.*, 1981; Cho *et al.*, 1984) which can accommodate large hydrophobic residues in S3/S4 (Bode *et al.*, 1989; Stubbs *et al.*, 1995; Brandstetter *et al.*, 1996).

These results clarify the role of some important active site determinants of fIXa and identify origins of fIXa's poor amidolytic properties. In the future, introducing these mutations into full-length fIXa will be useful in studying interactions with natural substrates and the cofactor, particularly in elucidating the fVIIIa-mediated increase in reactivity of fIXa towards fX. Additionally, these mutants enable engineering of fIXa molecules with increased reactivity. These are probably of therapeutic relevance, for example as substitutes in cases of haemophilia B, allowing reduced dosage through increased catalytic potency. Also, altered enzymatic properties might be desirable, as in the case of tPA (Madison, 1994) and thrombin (Gibbs *et al.*, 1995; Dang *et al.*, 1997).

Materials and methods

Materials

N-methoxycarbonyl-D-norleucyl-Gly-Arg-4-nitranilid acetate (MOC-D-Nle-Gly-Arg-pNA), *N*-methylsulfonyl-D-Phe-Gly-Arg-4-nitranilid acetate (MS-D-Phe-Gly-Arg-pNA) and human antithrombin III (ATIII) were purchased from Boehringer Mannheim (Germany); Q-Sepharose ff, heparin–Sepharose CL-6B and benzamidine–Sepharose 6B were from Pharmacia (Uppsala, Sweden). *p*-Aminobenzamidine (PABA), porcine heparin (grade I-A) and crude Russell's viper snake venom (RVV) were obtained from Sigma (Deisenhofen, Germany). The factor X activator RVV-X was purified as described (Esmon, 1973). 4-Nitrophenyl-4-

guanidiniumbenzoate (4-NPGB) and benzamidine were from Merck (Darmstadt, Germany). Purified human α -thrombin and human GLA-domainless fXa were a generous gift from Dr Wirthensohn (Boehringer Mannheim). Restriction enzymes and enzymes used for polymerase chain reaction (PCR) and cloning were from Boehringer Mannheim and New England Biolabs (Schwalbach, Germany). All other materials were of the highest grade available commercially.

Bacterial strains and plasmids

Cloning and expression of proteins was done in the *E. coli* K12 strain UT5600 (Grodberg and Dunn, 1988) carrying the lacI^q repressor plasmid pUBS520 (Brinkmann *et al.*, 1989). The plasmid pSA (Kopetzki *et al.*, 1993) was used for expression of recombinant proteins.

DNA manipulation techniques

All techniques used for amplification, mutagenesis and cloning of DNA followed published protocols (Sambrook *et al.*, 1989). Commercial kits were used according to the manufacturer's instructions.

Construction of recombinant proteins

The rf10 and rf9 variants encoded the second EGF domain, the activation peptide and the catalytic domain of human fX and human fIX. rf10 comprises residues Rn86 to Kn435 (n refers to the natural protein numbering), and rf9 comprises residues Dn85 to Tn415. DNA fragments encoding rf10 and rf9 were amplified by PCR using human liver cDNA constructed in the phage vector Lambda ZAP II (Stratagene, La Jolla, CA, USA) as template and the oligonucleotides 1 and 2 (Table IV) for rf10 and the oligonucleotides 8 and 9 for rf9 as primer. The PCR fragments for rf10 and rf9 were inserted into pSA using the restriction sites for *Eco*RI and *Hind*III, and *Nco*I and *Hind*III, respectively. The resulting plasmids for expression of rf9 and rf10 are referred to as pf9 and pf10. All mutant genes were constructed by PCR using pf9 or pf10 as template and oligonucleotides carrying the desired mutation as primer (Table V). The PCR fragments were cloned into pf10 or pf9 with a restriction site next to the mutation to be introduced and a convenient restriction site at some distance. In cases where no singular restriction site was closer than 50 bases to the site of mutation, a unique restriction site was created by changing the DNA sequence without altering the amino acid sequence. Two PCR fragments, carrying the newly created restriction site, were cloned into pf10/f9 in a single step by a three-fragment ligation. Multiple mutants were constructed by PCR on plasmids already carrying one or more mutations. All DNA segments amplified by PCR were verified by DNA sequencing using the dideoxy chain termination method on a 370A DNA Sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA).

Expression of recombinant proteins

A 60 ml portion of an overnight culture of *E. coli* UT5600 harbouring the lacI^q repressor plasmid pUBS520 and one of the expression plasmids pf9/f10/mutants was diluted into 3 l of 2× YT medium (2% tryptone, 1% yeast extract, 0.5% NaCl) containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Cells were grown at 37°C with shaking. At an OD₅₅₀ of 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce expression. After incubation for 4–6 h at 37°C, the cells were harvested by centrifugation at 6000 g and stored at –20°C.

Purification of inclusion bodies

The cell pellet was resuspended in 75 ml 50 mM Tris, pH 7.2. The suspension was incubated for 30 min at 0°C with 0.25 mg/ml lysozyme. After adding 150 µl of 1 M MgCl₂ and 750 µg DNase I (Boehringer Mannheim), cells were disrupted in a French pressure cell (Amico, Urbana, IL, USA) and then incubated for 30 min at 25°C to digest the DNA. After adding 38 ml 50 mM Tris, pH 7.2, 60 mM EDTA, 1.5 M NaCl, 6% Triton X-100 and incubating for an additional 30 min, the inclusion bodies were recovered by centrifugation for 30 min at 25 000 g.

Folding of recombinant proteins

The inclusion bodies were solubilized in 6 M guanidinium HCl, 100 mM Tris, pH 8.2, 20 mM EDTA, 150 mM oxidized glutathione and 15 mM reduced glutathione for 3 h at 25°C to give a final protein concentration of 5 mg/ml. After adjusting the pH to 5.0, the solubilized protein was dialysed twice for 24 h against 100 vols of 6 M guanidinium HCl, 20 mM EDTA, pH 5.0 at 4°C. Folding was initiated by diluting the solubilized protein in 100 vols of 50 mM Tris, 0.5 M arginine, 1 mM EDTA, 20 mM CaCl₂, 0.5 mM cysteine, pH 8.5 at 4°C followed by incubation of 2–3 days at 4°C.

Table IV. Oligonucleotides used for the construction of recombinant proteins

1	5'-AAAAAAGAATTCATTAAAGAGGAGAAATTAATGCGGAAGCTCTGCAGCCTGGACAAC-3'
2	5'-AAAAAAAGCTTCATTACTTGGCCTTGGGCAAGCCCTGGT-3'
3	5'-AAAAAACACGAGGTGGAGGTGGTCATCAAGCACAACCGGTTCAATGCAGCTATTAATAAGTATGACTTCGACATCGCCGTGC-3'
4	5'-AAAAAAGTCGACGCTGATGACGAGAAGACGGGGATTGTGAGCGGCTTCGGGCGCGTTTCCACAAGGGCCGGCAGTCCACCAG-3'
5	5'-AAAAAAGTCGACTCGGCCAGTCACGCTCGGGG-3'
6	5'-AAAAAAGTGACCTTGGTGTAGATCCCGTACTTCCCTTACGGGCGCAGCCCTCTCCCAGCTAATGATGCCTGTCCAGGAGTAGGTGTC-3'
7	5'-AAAAAAGTGACCTTGGTGTAGATCCCGTACTTCCCTTACGGGCGCATTCTCTCCCAGCTGACGATGCC-3'
8	5'-AAAAAACCATGGATGTAACATGTAACATTAAGAATGGCA-3'
9	5'-AAAAAAAGCTTCATTAAGTGAGCTTTGTTTTTCTTAATC-3'
10	5'-AAAAAATTCGAATTATTCCTCACCACAACCTACACCAAGAAACCTACAACCATGACATTGCCCTTCTGG-3'
11	5'-AAAAAACGCGCTCTCCACACCCACGAGAAAGGGAGATCAGCTTTAGTTCTTCAG-3'
12	5'-AAAAAACGCGTCCCCAGCCACTTACATAGCCAG-3'
13	5'-AAAAAACTAGTTTCTTAAGTGAATTGTGAGCTGGGGTGAAGAGTGTGCAATGAAAGGCAAATATGG-3'
14	5'-AAAAAACTAGTTTCTTAAGTGAATTATAGCTGGGGTGAAGGTTGTGCAATGAAAGGCAAATATGG-3'
15	5'-AAAAAACTAGTCCCTTCCACTTCAGTAACATGG-3'
16	5'-AAAAAACTAGTTTCTTAAGTGAATTGTGAGCTGGGGTGAAGGTTGTGCAATGAAAGGCAAATATGG-3'

Table V. PCR and cloning strategy using the indicated templates (boxes), oligonucleotide primer (arrows, listed in Table IV) and restriction endonucleases

	PCR Amplification	Template	Cloned into
pf10		human liver cDNA	pSA
pf10-99loop(f9)		pf10	pf10
pf10-148loop(f9)		pf10	pf10
pf10-V213I		pf10	pf10
pf10-G219E		pf10	pf10
pf9		human liver cDNA	pSA
pf9-99loop(f10)		pf9	pf9
pf9-148loop(f10)		pf9	pf9
pf9-I213V		pf9	pf9
pf9-E219G		pf9	pf9
pf9-I213V-E219G		pf9	pf9
pf9-148loop(f10)-I213V-E219G		pf9.213V-E219G	pf9
pf9-99loop(f10)-148loop(f10)-I213V-E219G		pf9-148loop(f10)-I213V-E219G	pf9

Purification of folded recombinant proteins

The folding solution (1–2 l) was concentrated on a Filtron Minisette equipped with an Omega10K membrane (Filtron, Karlstein, Germany) to a final volume of ~100 ml. The concentrated sample was dialysed for 20 h against 10 l of 20 mM Tris, 50 mM NaCl, pH 8.0 at 4°C. Precipitated protein was removed by centrifugation for 15 min at >25 000 g. The supernatant was loaded on a 1.5×8.5 cm column containing 15 ml Q-Sepharose ff equilibrated with 20 mM Tris, pH 8.0, and 50 mM NaCl. Recombinant protein was eluted with a linear gradient from 50 to 500 mM NaCl in 20 mM Tris, pH 8.0 and further purified by heparin affinity chromatography as described (Bajaj *et al.*, 1981). Proteins were stored at –80°C until use.

Activation of recombinant proteins

All proteins were activated using RVV-X (Rezaie *et al.*, 1993; Lenting *et al.*, 1995) as follows. One mg/ml of recombinant protein was incubated for 2–3 h (rf10) or 16–20 h (rf9) with 1 µg/ml (rf10) or 10 µg/ml (rf9) RVV-X in 20 mM Tris, 150–200 mM NaCl, 10 mM CaCl₂, pH 8.0, at 25°C. rf10a was purified further by affinity chromatography on benzamidine-Sepharose 6B as described (Krishnaswamy *et al.*, 1987). This method was found to be inappropriate for rf9a, rf9a mutants and

low-activity mutants of rf10a. In these cases, RVV-X and not fully activated proteins were removed by negative chromatography on Q-Sepharose ff, making use of a shift in the isoelectric point upon removal of the acidic activation peptide. Only correctly activated proteins passed the column.

Active site titration

The concentration of active sites was determined using ATIII and heparin as described (Lozier *et al.*, 1990). 5–10 µM enzyme was incubated with various concentrations of ATIII ranging from 0.5 to 5 µM in 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM CaCl₂, 2 mg/ml BSA, 5 µg/ml heparin. After 30 min at 25°C, the residual activity was measured in an EAR 400 AT microplate reader (SLT, Austria) using MOC-D-Nle-Gly-Arg-pNA as substrate. The concentration of ATIII was determined by titrating 4-NPGB-titrated α-thrombin.

Amidolytic assays

Experiments were performed in 50 mM Tris, 150 mM NaCl, 5 mM CaCl₂, 0.1% PEG 8000, pH 8.0 at 25°C in a temperature-controlled Varian Cary 1E dual beam spectrophotometer. Substrate concentrations ranged from 30 µM to 8 mM. Enzyme concentrations were between

2 and 200 nM. Reactions were started by adding 50 µl of enzyme to 750 µl of preincubated buffer and substrate. The change in absorption was recorded typically for 60 s at 405 nm. Kinetic parameters were calculated by non-linear curve fitting the initial rates to the Michaelis–Menten equation using the SigmaPlot software (Jandel GmbH, Erkrath, Germany). Inhibition constants of PABA were measured by competition with MOC-D-Nle-Gly-Arg-pNA. A 6×6 matrix was created by varying the concentration of MOC-D-Nle-Gly-Arg-pNA (50 µM to 1 mM) and PABA (0–10 mM) by repeated dilution by a factor of 2. The highest concentration was usually 5- to 10-fold the K_m (MOC-D-Nle-Gly-Arg-pNA) or K_i (PABA). 150 µl of enzyme in double-concentrated buffer was added to 150 µl MOC-D-Nle-Gly-Arg-pNA and PABA in H₂O in a 96-well microtitre plate. The change in absorption was measured in an EAR 400 AT ELISA reader (SLT, Austria). Kinetic data were calculated by global non-linear least square fitting of the reaction rate v to $v = e_T k_{cat} [S]/(K_m(1+[I]/K_i)+[S])$, where e_T , $[S]$ and $[I]$ denote the total concentration of enzyme, substrate and the PABA, respectively.

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