Directed Glycosylation of Human Coagulation Factor X at Residue 333

INSIGHT INTO FACTOR Va-DEPENDENT PROTHROMBIN CATALYSIS*

Bernard C. Cook‡, Amy E. Rudolph‡, Ravi G. Kurumbail, Rhonda Porche-Sorbet‡, and Joseph P. Miletich‡**

From the 1Division of Laboratory Medicine, Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 and the 2Division of Biochemistry and Molecular Biology, Pharmacia Corporation, St. Louis, Missouri 63110

Based on homology, amino acids 326–336 (143–154 in chymotrypsin numbering) of factor X (fX) comprise a flexible surface loop, which is susceptible to self-proteolysis and influences substrate catalysis. To investigate the role of this autolysis loop in fX function, a recombinant variant with a new site for asparagine-linked glycosylation has been produced by changing glutamine 333 to asparagine. Q333N fX is activated normally by factor VIIa and tissue factor, factors IXa and VIIIa, and Russell’s viper venom. Proteolysis of the loop is prevented by the mutation. Reactivity of the free enzyme toward substrates and inhibitors is attenuated 4–20-fold; relative to wild type fXa, Spectrozyme XaTM hydrolysis is 25%, inhibition by antithrombin III and the tissue factor pathway inhibitor is ~20%, and prothrombin activation in the absence of the cofactor Va is only 5%. Surprisingly, activities of the variant and wild type enzymes are equivalent when part of the prothrombinase complex. N-Glycanase cleaves the new oligosaccharide from Q333N fXa leaving aspartic acid. Q333D fXa is ~1.6-fold more reactive with Spectrozyme XaTM, antithrombin III and tissue factor pathway inhibitor, and prothrombin than its glycosylated counterpart, Q333N fXa, but still quite abnormal relative to wild type fXa. Like Q333N fXa, Q333D fXa is fully functional as part of the prothrombinase complex. We conclude that Gln-333 is geographically close to a site of proteolytic degradation but not to activator, cofactor, or membrane binding sites. Mutation of Gln-333 impairs catalytic function, but given normal prothrombin activation by the complexed enzyme, the importance of Gln-333 for catalysis is not manifest in the prothrombinase assembly, suggesting a conformational change in complexed fXa.

Human coagulation factor X (fX), a glycoprotein zymogen of $M_w \sim 58,000$, has a pivotal role in blood coagulation. The enzyme (fXa) is a trypsin-like serine protease that catalyzes the conversion of prothrombin to thrombin. The protein can be activated by factor VIIa/tissue factor (fVIIa/TF), factor IXa/factor VIIIa (fIXa/fVIIIa), or the snake venom-derived X-coagulant protein (XCP). Its subsequent catalytic activity has been well characterized using highly purified components. Similarly, inhibition of fXa (by antithrombin III (ATIII) and the tissue factor pathway inhibitor (TFPI) among others) is well documented. Prothrombin activation by fXa is accelerated up to 300,000-fold by its cofactor, factor Va, on a suitable lipid surface (1). Platelets constitute this assembling surface in vivo, whereas synthetic liposomes can provide a convenient model in experimental systems. To identify the regions of the fX(a) molecule that mediate biologic interactions of the protein, we have optimized an expression system to produce recombinant fX and systematically probe specific functional consequences of natural and directed mutations. Herein, we report the properties of a fX protein (RTHEKGRQOSTR→RTHEKGRNSTR) designed to have a new N-linked carbohydrate at residue 333 (151 in chymotrypsin numbering) in the serine protease domain. Targeted glycosylation offers a number of features as a mutational approach: 1) a carbohydrate side chain is a convenient size to mask selected regions of the fX molecule, 2) the success of the intended mutation is easily evaluated by altered migration on SDS/PAGE, and 3) the carbohydrate can be removed with the enzyme N-glycanase, allowing an assessment of the contribution of the new carbohydrate itself to any functional consequences. Human fXa catalyzes the cleavage of at least two peptide bonds in its own heavy chain in reactions that are accelerated by negatively charged phospholipid surfaces. The faster cleavage liberates a 2.3-kDa glycopeptide from the carboxyl terminus creating a smaller enzyme, fXa$b$, that is functionally very similar to the intact enzyme, fXa$g$ (2). The slower cleavage is within the 326–336 autolysis loop and produces fXay, a species that is an additional 11–12 kDa smaller by SDS/PAGE analysis and that has little or no catalytic activity (3). We reasoned that a new carbohydrate side chain at residue 333 might prevent degradation within the autolysis loop. Additionally, we predicted that an added carbohydrate residue in this region might impair catalytic activity based on the proximity of the region to the active site of the enzyme (3–5). This last prediction proved true for the free enzyme, but we did not anticipate that the

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‡ To whom correspondence and requests for reprints should be addressed: Clinical Laboratory Science, P. O. Box 1881, Marquette University, Milwaukee, WI 53201. Tel.: 414-288-3404; Fax: 414-288-5847; E-mail: bernard.cook@marquette.edu.

¶ Present address: Div. of Cardiovascular and Metabolic Disease, Pharmacia Corporation, St. Louis, MO 63110.

** Present address: Merck & Co., Inc., West Point, PA 19486.

1 The abbreviations used are: fX, factor X; ATIII, antithrombin III; TFPI, tissue factor pathway inhibitor; XCP, X-coagulant protein; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylycerine; PS, phosphatidylserine.
enzymatic efficiency of the mutant enzyme toward prothrombin would be normalized when the mutant enzyme is in the prothrombinase complex.

Others have shown that mutations designed to induce N-linked glycosylation at selected residues can be a useful means of surveying molecular regions of proteins (6–8). We herein demonstrate that induced glycosylation is useful in characterizing the contribution to the function of a specific region of FXα.

MATERIALS AND METHODS

Reagents and Chemicals—Spectrozyme THF™ (H-d-hexahydrotricosyl-t, t, t-arginine-p-nitroanilide diacetate) and Spectrozyme Xa™ (methyleneoxycarbonyl-h-hexahydrotricosyl-t, t, t-arginine-p-nitroanilide diacetate) were obtained from American Diagnostics, Greenwich, CT. Organic solvents were high pressure liquid chromatography grade. All other reagents and chemicals were of highest quality available commercially.

Phospholipid Vesicles—Egg yolk phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were purchased from Sigma. PC:PS (3:1) vesicles of nominal 100-nm diameter were made by membrane extrusion (9) in 10 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM CaCl2, at a concentration of 1.0 mM total lipid using the Liposofast Basic® device (Avestin, Ottawa, Canada). Immunoglobulins—Affinity-purified caprine anti-mouse Ig conjugated to horseradish peroxidase was obtained from Jackson ImmunoResearch (West Grove, PA). Murine monoclonal antibodies specific for human FX have been developed by standard methods and used for identification, quantitation, and purification of FX and are described elsewhere (10).

Proteins—Prothrombin was purified from human plasma by barium citrate adsorption/elution followed by column chromatography using QAE-Sepharose™ (11), sulfated dextran beads (12), and Mono Q™ FPLC. Following purification, prothrombin was concentrated (>1 mg) and immunodepleted of residual FX (<1.5 μg/ml) by passage through a column containing immobilized antibody 3448.20. ATIII was purchased from Kabi Pharmacia Diagnostics (Piscataway, NJ), Factors V, VA, VIIa, and IXa from human plasma were purchased from Hematologic Technologies (Essex Junction, VT). Thrombin was prepared by cation exchange chromatography after activation of human prothrombin with Taipan snake (Oxyuranus scutellatus) venom. Porcine FX was obtained from Porton Products (Agoura Hill, CA) and was further purified (13) using an immobilized antibody (W-3, a generous gift from Dr. David Fasse). Innovin™, a lipitated, recombinant tissue factor, was obtained from Baxter Diagnostics (Deerfield, IL). XCP was purified from Russell's viper venom (14). Recombinant TFPI was a gift from The Monsanto/Searle Company (St. Louis, MO). SDS/PAGE was performed by the method of Laemmli (15). Proteins were stained directly using Coomassie Blue or were transferred to nitrocellulose (0.45 μm), probed using antibodies described above, and visualized with an immunochromiluminéscent kit (Amersham Pharmacia Biotech). FX was purified from either plasma or culture media as described previously (16).

Mutagenesis, Transfection, and Cell Culture—The construction, transfection, and expression of wild type and mutant FX in human kidney cells (293 cells, American Type Culture Collection, CRL 1573) have been described elsewhere (10, 17). The expression vector (ZMB3) was a gift from Dr. Don Foster.

Indication of Proteins—FX was labeled with 125I using prelabeled Bolton-Hunter reagent (Amersham Pharmacia Biotech) in a modified procedure. After evaporation of the organic solvent, a buffered solution of FX (<10.0 μM, pH 8.5) was added to the vial and reacted with the Bolton-Hunter reagent for 15 min on ice. The unbound reagent was then reacted with 0.2 ml glycerine in the same buffer for 5 min on ice. Specific activity was typically ~5 × 10^16 Bq mol^-1.

Activation of FX—As detailed elsewhere (10), both wild type and Q333N FX (150 nM in reaction buffer, 100 mM NaCl, 2.5 mM CaCl2, and 10 mM Hepes, pH 7.5) were tested as substrates for three different activators: 1) 20 pmol VIIa and Innovin™ (500 pmol lipitated recombinant tissue factor); 2) 400 pmol IXa, 2 units ml^-1 VIIa (activated with 1.0 unit ml^-1 thrombin exactly 5 min before the start of the assay), and 20 μM PC:PS vesicles; or 3) 50 pmol XCP. These conditions represent sub saturating concentrations of activators.

Inhibition by ATIII or TFPI—Wild type and Q333N FX (150 nM in reaction buffer) were fully activated to the enzyme form with excess XCP (1.0 μM). The enzyme (diluted 300-fold to 0.5 μM) was then co-reacted with Spectrozyme Xa™ (100 μM) and either ATIII or TFPI, and appearance of the chromophore, p-nitroanilide acetate, was monitored over time at 405 nm. ATIII concentrations were varied from 0 to 8 μM. Primary data were fitted using the Marquardt-Levy algorithm (SigmaPlot, Jandel Scientific, Corte Madera, CA) to an exponential equation of the form,

\[ A = A_0 (1 - e^{-kt}) + A_r \]  

where \( A \) is the absorbance, \( A_0 \) is the amplitude of the curve, \( k \) is the apparent first order rate constant with units of s^-1, \( t \) is time in seconds, and \( A_r \) is the initial absorbance. Each calculated apparent rate constant (\( k_{act} \)) was then plotted as a linear function of the effective inhibitor concentration, and the resulting slope was calculated as the apparent second order rate constant. Experiments designed to monitor the inhibition of Xa by ATIII (0–10 μM) in the presence of sub saturating concentrations of heparin (2.5 milliunits ml^-1) or by TFPI (0–50 μM) were similar except that the primary data were fitted to an equation that accounts for the terminal rate attributed to uninhibited enzyme (18, 19).

\[ A = v_f t + ((v_f - v_i)A_r(1 - e^{-k_A t}) + A_i \]  

where \( v_f \) and \( v_i \) represent the initial and terminal rates of hydrolysis, respectively.

Thrombin Generation—The rates at which wild type and Q333N FXa can activate prothrombin to thrombin in reaction buffer were compared using established methods (10) and several different reagent compositions. The general conditions tested included: 1) 10 mM Xa with 0–450 μM prothrombin, 2) 0–1.0 mM FXa with 1.0 μM prothrombin and 10^6 ml^-1 washed platelets (preactivated with 0.5 unit ml^-1 thrombin), and 3) 0–200 pmol FXa with 1.0 μM prothrombin, 500 pmol tIVa, and 20 μM PC:PS vesicles. For each series of reactions, initial rates of thrombin generation were plotted as a function of the component being varied. These data were fitted (SigmaPlot, Jandel Scientific, Corte Madera, CA) to the hyperbolic equation,

\[ v = V_{max} [S]/(K_{app} + [S]) \]  

where \( v \) is the observed initial rates of thrombin formation, \( V_{max} \) is the maximal initial rate of thrombin formation, \( K_{app} \) is the concentration of the component being varied in the experiment, and \( K \) is the apparent concentration of the variable component required to reach half-maximal thrombin formation under the conditions specified.

Spectrozyme Xa™—To test activity against the synthetic substrate, 1.0 mM wild type or Q333N FXa was reacted with Spectrozyme Xa™ at concentrations of 0 to 500 μM. The initial rate of hydrolysis of Spectrozyme Xa™ was related to the substrate concentration using the hyperbolic function described above to estimate \( K_m \) and \( V_{max} \) and to calculate \( k_{cat} \) (Eq. 1AIXa).

N-Glycanase—One-quarter unit of recombinant N-glycanase (Genzyme, Cambridge, MA) was incubated with wild type or Q333N FXa (0.7 μM) at 27 °C for 18 h, after which the proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose for immunodetection. The N-glycanase-treated enzymes were also tested for activity against Spectrozyme Xa™ and prothrombin and for inhibition by ATIII and TFPI as described above.

RESULTS

Q333N FX Is Expressed with Additional Carbohydrate—Q333N FX has decreased electrophoretic mobility on SDS/PAGE compared with the wild type protein (Fig. 1A), consistent with the additional mass of a new oligosaccharide side chain. Under reducing conditions the difference between wild type and mutant proteins is more evident, as is the increased microheterogeneity of its heavy chain. A small fraction of the mutant protein co-migrates with wild type FX and may not be glycosylated at position 333. N-Glycanase (peptide-N-acetyl-β-glucosaminylasparagine amidase from Oxyuranus scutellatus) hydrolizes the β-aspartyl-glucosamine bond of N-linked oligosaccharides, which results in deamidation of the asparagine residue to aspartic acid (20, 21). Following treatment with N-glycanase, wild type and Q333N FXa co-migrate (Fig. 1B). There is no change in wild type FXa with N-glycanase treatment because all of its N-linked carbohydrate is on the 52-kDa acid peptide released by activation. The activation peptide itself is >50% carbohydrate and migrates anomalously
Q333N FX Is Activated Normally—Both wild type and Q333N FX (150 nM) quantitatively release their activation peptides when incubated with XCP (2.0 nM) as assessed by a two-site (light chain/activation peptide) immunoassay (data not shown). Further, no residual wild type or mutant FX zymogen is detectable by immunoblot after 60 min (sensitivity <0.5%, data not shown). Both enzymes hydrolyze Spectrozyme Xa TM (Fig. 2), but the overall catalytic efficiency of Q333N FXa is only 25% of the wild type enzyme with equal impact on $K_m$ and $k_{cat}$ (131 $\mu$M and 3.1 min$^{-1}$ for Q333N FXa compared with 65 $\mu$M and 6.2 min$^{-1}$ for the wild type enzyme). Initial rates of hydrolysis of Spectrozyme Xa TM, corrected for the catalytic efficiency, were used to calculate initial rates of activation of FX by submaximal concentrations of three different activators, fVIIa/TF, FXa/ fVIIa, and XCP. In each case there was no significant difference between wild type and Q333N FX with regard to either the rate or the extent of activation (data not shown). We conclude that activation of the mutated protein is normal.

Degradation to the γ Species Is Prevented by the Q333N Mutation—When 150 nM $^{125}$I-labeled wild type FX is activated by 2 nM XCP in the presence of 20 $\mu$M PC:PS vesicles for 45 min, autolysis at the $\beta$ and $\gamma$ cleavage sites (>90% and 30–40%, respectively) is readily apparent (Fig. 3, lane d). When $^{125}$I-labeled Q333N FX is treated in the same manner, no autolysis is seen. To distinguish whether Q333N FXa is a less efficient autolytic enzyme, a poorer substrate for autolysis, or both, 150 nM $^{125}$I-labeled Q333N FXa was first activated by XCP for 15 min in the presence of 40 $\mu$M PC:PS vesicles. It was then mixed with an equal volume of 150 nM unlabeled wild type or Q333N FXa, each having been fully activated just before use with XCP in the absence of phospholipid to minimize autolysis of wild type FXa. After an additional 45-min incubation, the reactions were stopped by the addition of SDS sample treatment buffer, and the effects on the $^{125}$I-labeled Q333N FXa were analyzed by autoradiography following SDS/PAGE. The increase in migration that results from cleavage at the $\beta$ site is very similar in magnitude to the decrease in migration caused by addition of a carbohydrate side chain at residue 333; i.e. FXa glycosylated at position 333 and cleaved at the $\beta$ site (Xaβγ) migrates the same as FXa not glycosylated at position 333 and not degraded (Xaa). The $^{125}$I-labeled Q333N FXa was not appreciably affected by the additional 45-min incubation with unlabeled Q333N FXa and PC:PS vesicles (Fig. 3, lanes a and c). In contrast, the added wild type FXa readily cleaved the $^{125}$I-labeled Q333N FXa at the $\beta$ site but was not able to cleave at the $\gamma$ site (Fig. 3, lane b). Mutation to Q333N results in 1) reduced activity of the mutant toward itself as a substrate at the $\beta$-cleavage site and 2) protection of the $\gamma$-cleavage site in the autolysis loop from proteolytic attack by either wild type or mutant enzyme.

**Inhibition by ATIII ± Heparin or TFPI Is Slower for Q333N FXa—**ATIII reacts much faster with wild type FXa than with Q333N FXa (Fig. 4). The second order rate constants, calculated as described under “Materials and Methods” for inhibition by ATIII in the absence of heparin, are $1.3 \times 10^5$ M$^{-1}$ s$^{-1}$ for wild type and $0.17 \times 10^5$ M$^{-1}$ s$^{-1}$ for Q333N FXa (13%). This reduced interaction of ATIII with the mutant enzyme is independent of heparin, and heparin accelerates the inhibition of both to roughly the same extent. In the presence of 2.5 milliunits ml$^{-1}$ heparin (less than half-saturating) the rate constants are $6.5 \times 10^5$ M$^{-1}$ s$^{-1}$ for wild type versus $1.6 \times 10^5$ M$^{-1}$ s$^{-1}$ for Q333N FXa (25%). In reactions with TFPI, the mutated enzyme is also inhibited more poorly than wild type; $1.1 \times 10^5$ M$^{-1}$ s$^{-1}$ for wild type versus $0.22 \times 10^5$ M$^{-1}$ s$^{-1}$ for Q333N FXa (20%).

**Activated Q333N Has Reduced Catalytic Efficiency for Prothrombin—**The ability of FXa to biologically process a native substrate, prothrombin, is also affected by the Q333N mutation. Prothrombin activation by wild type FXa is very slow in
the absence of accessory factors (the protein cofactor, fVa, and a negatively charged phospholipid surface), but Q333N fXa is 20 times slower still (Fig. 5), a substantial disruption in catalytic capacity. Platelets provide both a focusing surface and the cofactor (fVa) to accomplish a 300,000-fold increase in thrombin lytic capacity. Platelets provide both a focusing surface and the cofactor (fVa) to accomplish a 300,000-fold increase in thrombin lytic capacity.

Fig. 5. Thrombin generation by wild type and Q333N fXa in the absence of cofactor or phospholipid. Initial rates of prothrombin activation by 10 nM wild type (closed circles and solid line) or Q333N fXa (open circles and broken line). Data points are duplicate readings at each prothrombin concentration. Lines represent the best fit to the hyperbolic equation as described under "Materials and Methods."

In this system. However, in the presence of activated platelets, Q333N fXa activates prothrombin as well as the wild type enzyme with overall kinetics that are very similar (Fig. 6). Using 1.0 μM prothrombin and 108 platelets per ml, both the maximal rate of activation at saturating fXa (1.86 nM s−1 for wild type versus 2.01 nM s−1 for Q333N fXa in the experiment shown) and the concentration of enzyme required for half-maximal velocity (115 pm for wild type versus 91 pm for Q333N fXa in the experiment shown) are essentially the same and indistinguishable from plasma fXa tested under the same conditions (data not shown). The activities of both enzymes toward prothrombin are dramatically increased by the activated platelets, but the mutant is actually accelerated about 20 times more to reach that same level. Exhaustive immunodepletion of the prothrombin reagent as described was necessary to prevent thrombin formation from traces of plasma fX. A control, containing no added fXa but with the same amount of XCP used as the fX activator, was carried through the experiment in parallel and demonstrated no detectable thrombin activity.

To eliminate the possibility that some unidentified species derived from, or co-purified with, the platelets was responsible for nullifying the effect of the mutation on enzymatic activity, thrombin formation was measured using defined phospholipid vesicles and purified fVa (1.0 μM prothrombin, 20 μM PC:PS vesicles, 500 pm fVa). Again, the two enzymes have equivalent activities (Fig. 7). Neither the maximal rate of activation at saturating fXa (1.30 nM s−1 for wild type versus 1.27 nM s−1 for Q333N fXa) nor the concentration of enzyme required for half-maximal velocity (128 pm for wild type versus 115 pm for Q333N fXa) are detectably different.

Removal of the New Oligosaccharide with N-Glycanase Restores Some Activity—After activation, wild type fXa, in contrast to Q333N fXa, has no N-linked sugars. Treatment with N-glycanase should affect only the mutant enzyme and might restore function. The rates of hydrolysis of Spectrozyme-Xa™, inhibition by TFPI, and activation of prothrombin (without phospholipid or cofactor) are not changed by treatment of wild type fXa with N-glycanase. In contrast, Q333N fXa is only 40%, 30%, and 8% for Spectrozyme-Xa™ hydrolysis, inhibition by TFPI, and prothrombin activation,
respectively. Because the N-glycanase-catalyzed deglycosylation of asparagine leaves aspartic acid (Q333D fXa), this partial recovery suggests that there is specificity involved in modulation of the active site cleft by the autolysis loop in general and by residue Gln-333 in particular. Replacement of Gln-333 by Asp is, quantitatively, almost as detrimental as the addition of an entire N-linked carbohydrate side chain.

Q333D fXa, like its glycosylated precursor, activates prothrombin completely normally in the presence of activated platelets or factor Va and phospholipid. The side chain of this residue appears to have no specific role in regulating the activity of the prothrombinase complex despite its importance to the activity of the enzyme outside that context.

Structural Data Support a Role for the Autolysis Loop—Insight into the disrupted function that results from mutation of residue 333 can be gained from structural changes that occur when the enzyme is reacted with the fXa-specific inhibitor, DX-9065a. Because this synthetic amidino acid abates the activity of fXa but not thrombin (22), we speculated that the autolysis loop might be implicated, both in functional differences between fXa and thrombin and in structural differences between native and inhibitor-bound fXa. Indeed, the autolysis loop in the DX-9065a-bound fXa structure reported by Brandstetter et al. (5) adopts a conformation distinct from the partially ordered autolysis loop of the native enzyme (4). Fig. 8 depicts interactions of the autolysis loop with the serine protease domain of the enzyme. Similar to the interactions present in thrombin and factor VIIa, the N terminus of Ile-16 in the uninhibited fXa structure (4) forms an ion pair with the carboxylate of Asp-194. The autolysis loop of inhibitor-bound fXa is well ordered because of stabilizing interactions within the loop as well as those within the main body of the enzyme (Table I). A significant number of these associations involve the participation of the main chain nitrogen and carbonyl oxygen atoms of the autolysis loop residues. As a result of these interactions, the immediate environment of the N terminus (Ile-16) is altered somewhat. The ion pair that is observed between the positive charge on the N terminus and the carboxylate of Asp-194 is well ordered because of stabilizing interactions within the loop as well as those within the main body of the enzyme (Table I).

**FIG. 6.** Thrombin generation by wild type and Q333N fXa in the presence of activated platelets. Initial rates of activation of 1.0 μM prothrombin by 0–1.0 nM wild type (closed circles and solid line) or Q333N (open circles and broken line) fXa in the presence of 10^6 ml−1 thrombin-activated platelets are shown. Data points are duplicate measurements. Lines represent the best fit to the hyperbolic equation described under “Materials and Methods.”

**FIG. 7.** Thrombin generation by wild type and Q333N fXa in the presence of factor Va and PC:PS vesicles. Initial rates of activation of 1.0 μM prothrombin by 0–200 pM wild type (closed circles and solid line) or Q333N (open circles and broken line) fXa in the presence of 500 pM fVa and 20 mM PC:PS vesicles are shown. Data points are duplicate readings at each fXa concentration. Lines represent the best fit to the hyperbolic equation described under “Materials and Methods.”

**FIG. 8.** Relation of autolysis loop to N terminus of inhibitor-bound fXa. Solid lines represent hydrogen bonds that are unique to inhibitor-bound fXa and are not apparent in native fXa. See Table I and discussion under “Results.”

**TABLE I**

| Donor         | Acceptor       | Distance (Å) |
|---------------|----------------|--------------|
| Nh-Arg-143    | O-Gly-149      | 2.85         |
| N-Thr-144     | O-Gln-151      | 3.10         |
| N-Arg-154     | O-Ser-152      | 2.83         |
| N-Ile-16      | O-Thr-144      | 3.70         |
| O-Thr-144     | O-Ile-16       | 3.21         |
| N-Cys-220     | O-Glu-147      | 3.05         |
| N-Gln-151     | O-Gly-40       | 2.80         |
| N-Glu-147     | O-Glu-147      | 3.15         |

Data are from the Brookhaven Protein Data Bank (1HCG) and References 4 and 5.

DX-9065a. Because this synthetic amidino acid abates the activity of fXa but not thrombin (22), we speculated that the autolysis loop might be implicated, both in functional differences between fXa and thrombin and in structural differences between native and inhibitor-bound fXa. Indeed, the autolysis loop in the DX-9065a-bound fXa structure reported by Brandstetter et al. (5) adopts a conformation distinct from the partially ordered autolysis loop of the native enzyme (4).
and the oxyanion hole. Taken together, all evidence suggests that the conformational changes induced by the DX-9065a inhibitor might be implicated in the Q333N mutant and the Q333D deglycosylation product. Further, it appears that certain interactions between Glu-333 and regions of the heavy chain are essential for normal catalysis and that these interactions are altered, both in the inhibitor-bound enzyme and in Q333N. If true, this could explain the dramatically reduced catalytic efficiency of both 333 variants.

**DISCUSSION**

We targeted the autolysis loop of FX for glycosylation because it 1) has high surface accessibility, 2) is composed of unique amino acids compared with otherwise highly homologous serine proteases, 3) is susceptible to degradation, and 4) is spatially near and influences the activity of the catalytic cleft (3, 4, 23). The expectations that addition of a carbohydrate side chain to residue 333 would both impair the activity of the enzyme and prevent degradation at the autolysis loop were borne out. Because Q333D FXa is functionally quite similar to the glycosylated Q333N FXa, most of the change in specificity seems to be due to the loss of the glutamine side chain at residue 333 rather than to constraints imposed by the new carbohydrate side chain. Thus, the conformation of the autolysis loop that was observed by Brandstetter et al. (5) might represent a physiologically suboptimal conformation of FXa. It is also plausible that binding of the appropriate cofactors (e.g. FVa) in the region of the autolysis loop could induce a more favorable geometry to the catalytic site.

FXa-catalyzed activation of prothrombin is completely normalized in the presence of platelets or FVa/lipid. We speculate that thrombin generation can occur by two mechanistically different pathways depending on presence of cofactor/lipid. Further, the availability of FVa/lipid may be the main determinant for which pathway of prothrombin hydrolysis becomes important. We have presented evidence that the rate-limiting step in prothrombin hydrolysis in either of our replicable systems is not overall affinity for the substrate; FVa/lipid promotes equal activation of wild type and mutant. Rather, the rate of thrombin generation seems to be governed by the particular “presentation” of the substrate to the active site of the enzyme. As part of the prothrombinase complex, the mutant enzyme is no longer limited because the carbohydrate does not interfere with FVa-assisted prothrombin hydrolysis by this pathway. Whatever rate-limiting step now exists is equally restrictive for wild type and mutant enzymes.

Why should the presence of FVa/lipid equalize the enzymatic activity of the wild type and mutant enzymes? One possibility is that the conformation of the active site region changes when FXa binds to FVa/lipid in a manner that nullifies the effect of the added carbohydrate. Our data do not support this as the sole mechanism, however, as these studies failed to show enhanced hydrolysis of Spectrozyme Xa™ in the presence of FVa/lipid. Because the normalization of activity is restricted to prothrombin as the substrate, it is possible that prothrombin is made a better substrate by association with FVa/lipid such that the negative influence of the added carbohydrate is inconsequential. Consider FVa-bound prothrombin to be in a more restricted orientation with regard to the active site of FXa. In this more optimal orientation, contact of prothrombin with the catalytic site of FXa is a less random event than when FVa is not present, and the hindering effect of the added oligosaccharide is insignificant. Without FVa, however, there exists no “tether” to maximize and exploit FXa-prothrombin interactions. Only when FXa and FVa join as a complex on an immobilizing lipid surface are the synergistic effects of a focusing interface and the conformational induction of FVa-prothrombin realized.

The mutant Q333N FX is activated normally. Because the enzyme is resistant to proteolytic degradation and is inhibited about 5 times more slowly by ATIII and TFPI but still generates thrombin normally as part of the prothrombinase complex, it might have potential as a hemostatic agent based on an anticipated longer in vivo survival. Mutations in homologous regions of other serine proteases, particularly coagulation and complement factors that interact with cofactors that change their catalytic efficiency toward protein substrates, might well have similar consequences making it possible to further narrow their specificity and to extend their durability.

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