The 99 and 170 Loop-modified Factor VIIa Mutants Show Enhanced Catalytic Activity without Tissue Factor*

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To elucidate the functions of the surface loops of VIIa, we prepared two mutants, VII-30 and VII-39. The VII-30 mutant had all of the residues in the 99 loop replaced with those of trypsin. In the VII-39 mutant, both the 99 and 170 loops were replaced with those of trypsin. The $k_{cat}/K_m$ value for hydrolysis of the chromogenic peptide substrate S-2288 by VIIa-30 ($103 \text{ mm}^{-1} \text{s}^{-1}$) was 3-fold higher than that of wild-type VIIa ($30.3 \text{ mm}^{-1} \text{s}^{-1}$) in the presence of soluble tissue factor (sTF). This enhancement was due to a decrease in the $K_m$ value but not to an increase in the $k_{cat}$ value. On the other hand, the $k_{cat}/K_m$ value for S-2288 hydrolysis by VIIa-39 ($17.9 \text{ mm}^{-1} \text{s}^{-1}$) was 18-fold higher than that of wild-type ($1.0 \text{ mm}^{-1} \text{s}^{-1}$) in the absence of sTF, and the value was almost the same as that of wild-type measured in the presence of sTF. This enhancement was due not only to a decrease in the $K_m$ value but also to an increase in the $k_{cat}$ value. These results were in good agreement with their susceptibility to a subsite 1-directed serine protease inhibitor. In our previous paper (Soejima, K., Mizuguchi, J., Yuguchi, M., Nakagaki, T., Higashi, S., and Iwanaga, S. (2001) J. Biol. Chem. 276, 17229–17235), the replacement of the 170 loop of VIIa with that of trypsin induced a 10-fold enhancement of the $k_{cat}$ value for S-2288 hydrolysis as compared with that of wild-type VIIa in the absence of sTF. These results suggested that the 99 and the 170 loop structures of VIIa independently affect the $K_m$ and $k_{cat}$ values, respectively. Furthermore, we studied the effect of mutations on proteolytic activity toward S-alkylated lysozyme as a macromolecular substrate and the activation of natural macromolecular substrate factor X.

Coagulation factor VIIa (VIIa) is a plasma serine protease that is essential for the initiation of extrinsic blood coagulation (1). When tissue factor (TF) is expressed after injury of the vessel wall, it forms a complex with factor VIIa, and blood coagulation can be initiated. Forming a complex with TF markedly enhances the ability of VIIa to activate factors IX and X. In vitro, the formation of the active complex can be evidenced by measuring the esterolytic and amidolytic activities of VIIa (2, 3); these activities are also enhanced in the presence of soluble TF (sTF) and calcium ions (3–5).

Human zymogen VII is a single chain enzyme precursor with an NH$_2$-terminal Gla domain, followed by two EGF-like domains, EGF-1 and EGF-2, and a COOH-terminal serine protease domain. Through limited proteolysis of the Arg$^{152}$-trypsinogen peptidase bond [chymotrypsinogen numbering in brackets], zymogen VII is converted to a two-chain form enzyme, activated VII (VIIa), bridged by a disulfide bond (Cy$^{152}$–Cy$^{262}$), which is composed of a light chain (residues 1–152) with Gla, EGF-1, and EGF-2 domains, and a heavy chain with a serine protease domain (residues 153–406) (6). The crystal structures of the molecular complex of the active site occupying VIIa, with and without sTF, as well as the crystal structure of zymogen VII, are known (7–12). The shape of VIIa could be described as a tulip, with the catalytic domain as the flower, the light chain as the stem, and the Gla domain as the bulb. TF winds around the light chain (the stem) and the Gla domain (the bulb) of FVII.

Conversely, the catalytic domain of the coagulation proteases such as VIIa, IXa, Xa, and $\alpha$-thrombin have active sites and internal cores that are similar to those of trypsin. Although these protease domains have a highly homologous three-dimensional structure, they display significant differences in specificity and catalytic activity. Furthermore, each protease requires a specific cofactor to express enhanced catalytic activity and physiological function, which differs from digestive serine proteases such as trypsin and chymotrypsin. The serine protease domain consists of 12 $\beta$-strands and the loops between them. The $\beta$-strands form a scaffold of the catalytic domain, and their $\alpha$-carbon traces are comparable despite differences in amino acid residues. The individual proteases may be characterized by the surface loops of varying lengths and compositions (13, 14). For instance, in the case of $\alpha$-thrombin, the 60 loop (loops numbered according to the chymotrypsinogen numbering system) plays an important role in substrate specificity (15). In the cases of activated protein C, IXa, and Xa, substrate 2 is present in the 99 loop and regulates their substrate specificities (16–22). Comparisons of the three-dimensional structures of the active site regions of $\alpha$-thrombin, VIIa, IXa, Xa, and trypsin referred to the substrate specificities of these proteases (13, 23). Recently, Hopfner et al. (22) reported that replacement of some residues in the 99 loop of IXa conferred enhanced catalytic activity toward synthetic peptideyl substrates. Furthermore, the 170 loop of IXa has some interaction with cofactor factor VIII (24, 25). In $\beta$-thrypsin, the 60 loop, 99 loop, and

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§ The abbreviations used are: VIIa, activated coagulation factor VII; VII, zymogen factor VII; VIIa-W, activated wild-type factor VII; TF, tissue factor; sTF, soluble tissue factor (extracellular domain of TF; residues 1–218); APMSF, $p$-amidinophenylmethanesulfonyl fluoride hydrochloride; EGF, epidermal growth factor; TAP-lysozyme, S-3-(trimethylated amino)-propylated lysozyme; ELISA, enzyme-linked immunosorbent assay; $p$NA, $p$-nitroanilide; PEG, polyethylene glycol.

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170 loop have a major interface to form a tetramer, and this tetramerization is important for expressing activity (26). In this study, to investigate the function of the 99 loop of VIIa, which contains a substrate 2 and is located close to Asp1102, a member of a catalytic triad, we prepared two mutants and examined their enzymatic properties. One of the mutants had all of the residues in the 99 loop replaced with those of trypsin. Kinetic studies of these mutants in comparison with wild-type VIIa suggested that both the 99 and 170 loop structures of VIIa independently affect not only the $K_m$ and $k_{cat}$ values for peptide substrates, but also the substrate specificity of this enzyme, respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**—The materials used were as follows: S-2238 (H-o-Ile-Pro-Arg-p-nitroanilide (pNA) dihydrochloride (2HCl)), S-2268 (pyruvoly-Pro-Arg-pNA), S-2238 (H-o-Phe-Pro-Arg-pNA), S-2303 (H-o-Pro-Ph-Pro-Arg-pNA2HCl), S-2765 (Z-o-Arg-Gly-Arg-pNA2HCl), S-2444 (pyruvoly-Gly-Glu-pNA), S-222 (benzoyl-Ile-Glu(GluGly-Na) and S-2403 (pyruvoly-Glu-Lys-pNA-HCl) were from Chromogenix AB, Stockholm; Chromozym-t-PA (MeSO$_2$-O-Phe-Gly-pNA) and Chromozym-X (MeO-Co-o-Ile-Gly-pNA-gamma) were from Roche Diagnostics, Basel; $p$-aminohippuric acid, phosphatidylcholine, phosphatidylserine, horse spleen 6-aminohexanoic acid (APSMF), and butyric acid were from Wako Pure Chemical Industries, Ltd., Osaka; benzamidine HCl was from Tokyo Chemical Industry Co., Ltd.; LipoFECTACE TM Reagent, GENETICIN ® (antibiotics G418), and a-minimum essential medium were from Invitrogen; ASF-104 medium was from Ajinomoto Co., Inc., Tokyo; fetal bovine serum was from HyClone; penicillin G potassium was from Banyu Pharmaceutical Co., Ltd., Tokyo; streptomycin sulfate was from Meiji Seika Kaisha, Ltd., Tokyo; vitamin K, polyethylene glycol 8000 (PEG), bovine serum albumin (fatty acid-free), phosphatidylcholine, phosphatidylserine, and $\beta$-casein were from Sigma; Asserachrom® VII:Ag (ELISA kit), rabbit anti-human factor VII serum was from Diagnostica Stago, Asnieres, France; anti-rabbit IgG labeled with alkaline phosphatase was from Daco; 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were from Kirkegaard & Perry Laboratories. All custom oligo-DNA primers were provided by Nippon Pharm Mills Co., Ltd., Tokyo. All other chemicals were of analytical grade or of the highest quality commercially available.

**Proteins**—Recombinant human $\alpha$TF was prepared as described (27), and plasma-derived human clotting factors VII, VIIa, X, and Xa were prepared as described (28–30). S-3 (Trimethylated amino)-propylated lysosome (TAP-lysosome) was a gift from Drs. O. Ueda and T. Imoto, Kyushu University (31).

**Preparation of VIIa Mutants**—The construction of VII mutants by PCR-based methods was performed as described previously (32). Sequences of primers for mutagenic PCR were as follows: 5'-AGGAGCT-ACGACAGAGAGCTGACACCACAGCCTGCTCTGTTG-3' (sense) and 5'-GACAGCGGAGTCTGATGCTAGAGCTTCTGCTGTAG-GTGCT-3' (antisense) for VII-39. This reaction was termin- ated by the addition of 5:1 volumes of 2× PCR buffer, followed by 15 cycles of 94°C for 1 min, 68°C for 2 min, and 72°C for 2 min. The VII mutant was eluted with 100 mM Tris-HCl, pH 7.2, containing 0.1 mM NaCl, 50 mM benzamidine-HCl, and 2 mM CaCl$_2$. After the VII mutant was loaded, the gel was washed with 1 ml of equilibration buffer at least 3 times. The VII mutant was eluted with 100 μl of 50 mM Tris-HCl, pH 7.2, containing 0.1 mM NaCl, 50 mM benzamidine-HCl, and 10 mM EDTA. Aliquots of 20 μl of each sample were subjected to SDS-PAGE and Western blotting analyses. Anti-human FVII polyclonal antibody (Diagnostica Stago) was used as the primary antibody, and anti-rabbit IgG labeled with alkaline phosphatase (Dako) was used as the secondary antibody, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazo- lium were used as the substrate for development.

**Expression and Purification of VII Mutants**—Expression, cell culture, and purification of the VII mutants were performed as reported previously (32), with some modifications. Each clone was cultured and expanded in 10–20 roller bottles. Forty eight hours before harvesting, the culture media were replaced with ASF-104 media supplemented with 50 μg/ml vitamin K, with or without 5% fetal bovine serum. The monoclonal antibody used for purification of the VII mutant did not react with fetal bovine serum potentially including bovine VIIa as a contaminant (data not shown). After harvesting, the conditioned media were mixed with 50 mM benzamidine HCl, with or without 0.1% BSA, and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatants (1.5–6 liters) were stored at −80°C. The frozen media were thawed and filtrated through a 0.45-μm membrane filter and concentrated to 400–500 ml by ultrafiltration with a hollow fiber system (Asahi Kasei, Tokyo). The concentrated media were mixed with 2 mM CaCl$_2$, and the pH was adjusted to 6 by adding 100 mM HEPES buffer, pH 6, and the resulting media were subjected to Ca$^{2+}$-dependent anti-human factor VII antibody, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were used as a standard to estimate the protein concentration. After confirming that the contents of γ-carboxygluta- mate in each of the VII mutants were the same as those of plasma-derived VII, using the alkaline hydrolysis method (33), we then used the VII mutants for all experiments.

**Preparation of VIIa Mutants**—Activations of the zymogen VII mutants were achieved at 37 °C for 1–16 h by adding a 1:25:1:100 molar ratio of plasma-derived Xa in 50 mM Tris-HCl, pH 8, containing 0.1 mM NaCl, 0.1% PEG 8000, and 10 mM CaCl$_2$, and with or without phospholipid vesicles. Phospholipid vesicles (phosphatidylcholine/phosphatidy- lserine/cholesterol, 7:2:1:0.1) were prepared as described (34). This reaction was termin- ated by the addition of 5:1 volumes of 2× Tris-buffer, and centrifuged at 13,900 g for 15 min at 4°C. The pellet was derived from human plasma was used as a standard to estimate the protein concentration. After confirming that the contents of γ-carboxygluta- mate in each of the VII mutants were the same as those of plasma-derived VII, using the alkaline hydrolysis method (33), we then used the VII mutants for all experiments.

**Assays Using Synthetic Chromogenic Substrates**—All assays, including amidolytic of synthetic chromogenic substrates, TF dependence of VIIa mutants on amidolyis of S-2288, measurement of kinetic parameters for mutant VIIa-catalyzed amidolysis of S-2288, APMSF incorporation into VIIa mutants, and carbamylation of the $\alpha$-aminooxy group in VII mutants were performed as performed previously (32). Unless otherwise stated, kinetic experiments were carried out at 37°C with the following conditions: 50 mM Tris-HCl, pH 8.0, containing 0.1 mM NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000; 200 μl of sample in 96-well microplates (96 Polyisorp Nunc®Immuno Plate; NalgeNunc International Den- mark) measurement at 30°C with a SPECTRAmax plus® temperature-controlled microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Kinetics were measured by monitoring absorbance at 280 nm (λabs $\approx$ 280 nm) and monitored absorbance at 280 nm (λabs $\approx$ 280 nm) and monitored absorbance at 280 nm (λabs $\approx$ 280 nm) and monitored absorbance at 280 nm (λabs $\approx$ 280 nm).
was terminated by adding a final concentration of 20 mM EDTA in the ratio of VIIa mutant (1.27 nM; in the cases of VIIa-31 and VIIa-39, ranging from 50 to 800 nM) in concentrations (in the cases of VIIa-W and VIIa-30, ranging from 5 to 80 nM) with 50 mM Tris-HCl, pH 7.5, containing 0.1 mM NaCl, 5 mM CaCl₂, and 0.02% Tween 80 at 25 °C using a final concentration of 1 mM of S-2765, and the amount of Xa was above were sampled and diluted. Kinetic analyses were performed

The locations and amino acid sequences of the 99 and 170 loops in the protease domain of VIIa. Sequence comparisons of the 99 and 170 loops derived from serine proteinases of various species are indicated. VII, factor VII; IX, factor IX; X, factor X; PC, protein C; IIa, α-thrombin; try, trypsin. The crystal structure is cited from Protein Data Bank code 1DAN (7).

et al. (35), with some modifications. Briefly, 650 resonance units (RU) of sTF were immobilized on the sensor chip by means of an amine coupling method. Binding analyses were performed on the same chip repeatedly (regenerated with 50 mM EDTA between runs) at various protein concentrations (in the cases of VIIa-W and VIIa-30, ranging from 5 to 80 nM; in the cases of VIIa-31 and VIIa-39, ranging from 50 to 800 nM) in 50 mM Tris-HCl, pH 7.5, containing 0.1 mM NaCl, 5 mM CaCl₂, and 0.02% Tween 80 at 25 °C. The flow rate was 5 μl/min, and the association and dissociation phases were 7 and 15 min, respectively. Data were fitted by non-linear regression using a 1:1 Langmuir binding model and/or heterogeneous analyte-competition reactions model in BIAevaluation version 3.0.

Proteolysis of TAP-Lysozyme—Proteolysis of TAP-lysozyme (500 μg/ml, 31.7 μM) was carried out at 37 °C for 0.5–6 h by adding a 1:25 molar ratio of VIIa mutant (1.27 μM) with or without sTF (6.35 μM) in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM NaCl, 10 mM CaCl₂, and 0.1% PEG 8000. Aliquots of the reaction mixtures were withdrawn at various time intervals and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Steady-state Kinetics of Factor X Activation—The activation of factor X was carried out in the absence or presence of sTF. In the absence of sTF, 90 μl of a 400 nm concentration of each of the VIIa mutants was incubated with 10 μl of various concentrations of factor X ranging from 6 to 100 μM in 50 mM Tris-HCl, pH 8.0, 0.1% PEG 8000, 0.01% bovine serum albumin, and 100 mM NaCl, at 30 °C, for 30 min. The reaction was terminated by adding a final concentration of 20 mM EDTA. In the presence of sTF, 45 μl of an 8 mM concentration of each of the VIIa mutants and 45 μl of a 400 nm sTF were mixed and preincubated at 30 °C, for 10 min, adding 10 μl of various concentrations of factor X ranging from 0.5 to 25 μM. The reaction mixtures were incubated for 5 or 10 min, and the reaction was terminated by adding a final concentration of 20 mM EDTA. To determine the amount of activated factor X (Xa), aliquots of 10 or 50 μl of each of the reaction mixtures described above were sampled and diluted. Kinetic analyses were performed using a final concentration of 1 μM of S-2765, and the amount of Xa was then calculated by reference to a standard curve constructed using known amounts of Xa.

RESULTS

Construction and Expression of VII Mutants—The locations and amino acid sequences of the 99 and 170 loops in the protease domain of VIIa are shown in Fig. 1. Construction of VII mutants is also shown in Fig. 2. In mutant VII-30, all of the residues in the 99 loop were replaced with those of trypsin. In VII-39, both the 99 and 170 loops were replaced with those of trypsin. The cDNAs of these mutants and wild-type VII were stably transfected into CHO-K1 cells. The CHO-K1 cells expressing VII-31 and wild-type VII were obtained in our previous study (32). The time courses of the activation of each mutant in cell culture were estimated by Western blotting (Fig. 3). Wild-type VII was expressed at a level of 6 μg/ml and showed no activation. VII-30, the 99 loop-replaced mutant, was expressed at a level of 1.5 μg/ml and showed activation from the 3rd to the 4th day, as revealed by the appearance of heavy and light chains, but the intact molecule still remained on day 6. VII-31, the 170 loop-replaced mutant, was expressed to 1 μg/ml and showed activation from the 2nd day, and the intact molecule was almost completely activated on day 6. VII-39, both 99 and 170 loops, was expressed at less than 1 μg/ml and showed activation from the 2nd day. No intact molecule was seen on the 5th day, and degradation products were clearly detected on the 4th day. On SDS-PAGE, the bands of the heavy chains of VIIa-31 and VIIa-39 showed further mobility than those of VIIa-30 and VIIa-39, which exist on the heavy chains of both wild-type and VII-30, were detected by lectin blotting analysis (data not shown).

Preparation of VIIa Mutants—Activation of the purified VII mutants was performed under the conditions described under “Experimental Procedures.” As shown in Fig. 4, the autoactivation of VII-30 was slow. After 17 h of incubation, the zymogen VII-30 could still be observed. The activation of VII-30 by Xa was completed within 6 h, and the addition of phospholipids strongly enhanced this activation, which was completed within 15 min. On the other hand, the autoactivation of VII-39 was completed in about 60 min. This autoactivation was affected by calcium ions and pH. In cases where calcium ions were chelated by EDTA or media pH was 6, autoactivation of VII-39 did not occur within 60 min.
TF Dependence of Amidolytic Activities of VIIa Mutants—
The amidolytic activities of wild-type, VIIa-30, VIIa-31, and VIIa-39 toward S-2288, in addition to the effects of TF on these activities, were examined in the absence and presence of various concentrations of sTF. As shown in panels A and B of Fig. 5, sTF potentiated the amidolytic activities of all the mutants and wild-type VIIa in a dose-dependent manner. The data were subjected to the Hanes-Woolf plot ([sTF]/v versus [sTF] plot; according to Ref. 36) analysis to determine the apparent dissociation constants ($K_d^{(app)}$) for each mutant and wild-type VIIa complex with sTF (Fig. 5C). In VIIa-30 ($K_d^{(app)} = 14$ nM), the affinity for sTF was similar to that of wild-type ($K_d^{(app)} = 20$ nM); however, the affinities of VIIa-31 and VIIa-39 were reduced by about one order of magnitude ($K_d^{(app)} = 90$ and 360 nM), respectively.

Surface Plasmon Resonance Analyses of VIIa Mutants Binding to Immobilized sTF—The representative sensorgrams of each of the VIIa mutants are shown in Fig. 6. The data of VIIa-W and VIIa-30 could be comparatively well fitted by nonlinear regression using a 1:1 Langmuir binding model in BIAevaluation version 3.0. VIIa-30 associated about 2.4-fold slower with sTF ($2.4 \times 10^5$ M$^{-1}$ s$^{-1}$) than that of wild type. The resultant $K_d$ values were $3.2$ nM for VIIa-W and $9.6$ nM for VIIa-30. On the other hand, the data of VIIa-31 and VIIa-39, 170 loop modified mutants, could not be fitted by a simple 1:1 Langmuir binding model but were best fitted by a heterogeneous analyte-competition reactions model in BIAevaluation version 3.0. These results suggested that VIIa-31 exist in two different states, which are dominating low affinity state and a minor high affinity state to sTF. Consequently, the $K_d$ value of VIIa-31 was not concretely determined under these kinetic conditions. Similar results were observed in VIIa-39.

Kinetic Parameters for Mutant VIIa-catalyzed Amidolysis—The kinetic parameters for the hydrolysis of S-2288 catalyzed by VIIa mutants were determined (Table I). In the absence of sTF, the $K_m$ values of VIIa-30, -31, -39, and wild-type were 0.8, 2.3, 1.5, and 2.5 mM, respectively. These values of VIIa-31 was similar to that of wild type, whereas the values of VIIa-30 and VIIa-39 were decreased by about 2-fold. In contrast, the $k_{cat}$ value of VIIa-30 was similar to that of wild-type, whereas the values of VIIa-31 and VIIa-39 were 5–10-fold higher than that of wild-type. The $k_{cat}/K_m$ values of VIIa-30, VIIa-31, and VIIa-39 were higher than that of wild-type.
enhanced by 4-, 7-, and 18-fold higher than that of wild type, respectively. In the presence of sTF, the $k_{cat}/K_m$ value of wild-type was enhanced by about 30-fold and that of VIIa-30 showed a significant decrease in the $K_m$ value (0.4 mM), as compared with that of wild-type VIIa without sTF (2.5 mM). On the other hand, in the absence of sTF, VIIa-31 and VIIa-39 showed higher activities than wild-type, and sTF enhanced their activities ($V_{max}/K_m$) by about only 2-fold.

Incorporation of APMSF into VIIa Mutants—The formation of the catalytic site of the VIIa mutants was studied by examining the susceptibility to the inhibitor APMSF. As shown in Fig. 7, in the absence of sTF, the catalytic site formation of wild-type VIIa was not complete; thus, a high concentration of APMSF was needed for inactivation. After wild-type VIIa formed a complex with sTF, the catalytic site formed well; thus, the concentration of APMSF required for 50% inhibition (IC$_{50}$) was reduced from 2000 to 400 nM. In the case of VIIa-30, the concentration of APMSF needed for inactivation was similar to that of wild type (IC$_{50}$ = 1000 nM). On the other hand, in the cases of VIIa-31 and VIIa-39, the concentrations of APMSF needed for inactivation were reduced to 120 and 100 nM, respectively, suggesting that in these mutants the S1 site formation occurred effectively, even in the absence of sTF. After each VIIa-30, VIIa-31, and VIIa-39 formed a complex with sTF, the IC$_{50}$ values of each mutant were 10, 20, and 30 nM, respectively.

Substrate Specificity of VIIa Mutants—To examine the substrate specificities of these mutants, various chromogenic substrates were tested in the absence of sTF. As shown in Table II, all the mutants showed higher amidolytic activities than those of the wild type toward all of the substrates examined. The ratios of activities of VIIa-30/wild-type and VIIa-39/wild-type varied from 1 to 9, and 6 to over 50, respectively. These ratios became much higher when the substrates had a Gly residue at the P2 site. On the other hand, the ratio of activities of VIIa-31/wild-type varied from about 2 to 25, and this ratio became much higher when the substrates had a pyro-Glu residue at the P3 site.

Proteolysis of TAP-Lysozyme—Previous observations suggested that implantation of the trypsin sequence in the 99 and 170 loops of VIIa markedly enhanced catalytic activity. We examined whether such enhancement of VIIa catalytic activity results in the development of proteolytic action similar to that of trypsin, using protein substrates, such as S-alkylated lysozyme.
sozyme and casein. Fig. 8 shows the proteolysis of TAP-lysozyme by VIIa mutants. In the absence of sTF, no proteolysis was observed with wild-type VIIa, but slight proteolysis was observed with VIIa-31 and VIIa-39. Identification of the peptide fragments obtained by proteolysis with VIIa-39 indicated that the major cleavage sites were “Arg-X” contained in TAP-lysozyme, as expected (data not shown). In the presence of sTF, we observed significant enhancement of the proteolytic activity of all the mutants, especially of VIIa-30. The proteolysis of casein by these mutants and wild-type VIIa, in the absence and presence of sTF, showed similar results (data not shown).

**Activation of Factor X by VIIa Mutants**—The steady-state kinetic parameters for the activation of zymogen factor X were compared for VIIa-W, -30, -31, and -39 in the absence or presence of sTF (Fig. 9 and Table III). In the absence of sTF, the $K_m$ values on factor X activation of VIIa-W, -30, -31, and -39 were 17.4, 2.6, 1.9, and 2.1 $\mu M$, respectively. The $K_m$ values of VIIa-30, -31, and -39 were decreased by 10-fold compared with that of VIIa-W. In contrast, the $K_m$ value of VIIa-30 was similar to that of wild type, whereas those of VIIa-31 and VII-39 were 5- to 10-fold higher than that of wild type. The enhancement of the $k_{cat}$ values in the 170 loop-modified mutants, VIIa-31 and 39, were consistent with the data of amidolytic activity toward S-2288. The $k_{cat}/K_m$ values of VIIa-30, VIIa-31, and VII-39 were enhanced by 3-, 100-, and 40-fold higher than that of wild type, respectively. Ultimately, VIIa-31 showed the highest Xa generation as compared with those of VIIa-W, VIIa-30, and VIIa-39. There was a slight difference from the data of the amidolytic activity toward S-2288. There might be some differences in the substrate recognition mechanism between small chromogenic substrates and high molecular weight natural substrates, since a decrease in $K_m$ values of VIIa-31 and VIIa-39, 170 loop-modified VIIa mutants, were induced. On the other hand, in the presence of sTF, the $K_m$ values of VIIa-30, -31, and -39, and wild type were 0.40, 0.56, 0.25, and 1.1 $\mu M$, respectively. The activities ($k_{cat}/K_m$) of VIIa-W and VIIa-30 were enhanced by about 70,000- and 30,000-fold, respectively, when complexed with sTF. The $k_{cat}/K_m$ value of VIIa-30 was enhanced by about 1.5-fold higher than that of wild type. In contrast, the $V_{max}/K_m$ values of mutants VIIa-31 and VIIa-39 were decreased by about 10-fold from that of VIIa-W.

**TABLE I**

| Kinetic parameters for VIIa-W and mutants toward chromogenic substrate (S-2288) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | $K_m$ ($\mu M$) | $V_{max}$ (nmol s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $V_{max}/K_m$ (nmol s$^{-1}$) | $k_{cat}/K_m$ (nmol s$^{-1}$) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| VIIa-W                          | 2.52            | 262             | 2.62            | 104             | 1.04            |
| VIIa-30                         | 0.83            | 325             | 3.25            | 392             | 3.92            |
| VIIa-31                         | 2.26            | 1470            | 14.7            | 650             | 6.50            |
| VIIa-39                         | 1.50            | 2689            | 26.9            | 1793            | 17.9            |
| VIIa-W                          | 1.34            | 4064            | 40.6            | 3033            | 30.3            |
| VIIa-30                         | 0.37            | 3807            | 38.1            | 10289           | 102.9           |
| VIIa-31                         | 1.41            | 2047            | ND              | 1452            | ND              |
| VIIa-39                         | 1.32            | 4586            | ND              | 3474            | ND              |

In the presence of sTF, the $K_m$ values of VIIa-30, -31, and -39 were decreased by 3-, 100-, and 40-fold higher than that of wild type, respectively. In the presence of sTF, the $K_m$ values of VIIa-30, -31, and -39, and wild type were 0.40, 0.56, 0.25, and 1.1 $\mu M$, respectively. The activities ($k_{cat}/K_m$) of VIIa-W and VIIa-30 were enhanced by about 70,000- and 30,000-fold, respectively, when complexed with sTF. The $k_{cat}/K_m$ value of VIIa-30 was enhanced by about 1.5-fold higher than that of wild type. In contrast, the $V_{max}/K_m$ values of mutants VIIa-31 and VIIa-39 were decreased by about 10-fold from that of VIIa-W.

**DISCUSSION**

Significant enhancement of TF-independent amidolytic activity was induced by replacement of the 99 and/or the 170 loops of VIIa with the trypsin sequence. In comparison with wild-type VIIa, modification of the 99 loop (VIIa-30) induced the following changes: 1) some autoactivation in the expressing cell culture; 2) a decrease in the $K_m$ values toward S-2288 by about 3-fold in the absence and presence of sTF, respectively; 3) various degrees of enhancement of amidolytic activities toward all of the small substrates, and a change in the substrate specificity; 4) significant enhancement of the proteolysis of TAP-lysozyme in the presence of sTF; 5) a decrease in the $K_m$ values toward a natural substrate, factor X, by about 7- and 2-fold in the absence and presence of sTF, respectively; and 6) an enhancement of $k_{cat}/K_m$ toward factor X in the presence or absence of sTF. These observations suggested that TF-independent enhancement of the catalytic efficiency induced by the modification of the 99 loop of VIIa mainly causes a decrease in the $K_m$ value, resulting in an increase in the $k_{cat}/K_m$ value.

Modification of both the 99 and 170 loops (VIIa-39) induced the following changes: 1) the highest enhancement of autoactivation and further degradation in the expressing cell culture; 2) considerable autoactivation in the purified mutant; 3) a decrease in the apparent affinity for sTF by about one order of magnitude (Fig. 5); 4) a decrease in the TF-dependent enhancement of amidolytic activity; 5) a decrease in the $K_m$ value toward S-2288 by about 1.5-fold in the absence of sTF; 6) an increase in the $k_{cat}/K_m$ value toward S-2288 by about 10-fold in the absence of sTF; 7) an increase in the susceptibility to APMSF in the absence of sTF; 8) considerable enhancement of the
The 99 and 170 Loop-modified VIIa Mutants

9) various elevated degrees of enhancement of amidolytic activities toward all of the small substrates and a change in the substrate specificity; 10) a decrease in the $K_m$ value toward factor X by about 8-fold in the absence of sTF; and 11) an increase in the $k_{cat}$ value toward factor X by about 5-fold in the absence of sTF. These observations suggested that the changes observed with VIIa-39 were the sum of the changes of both VIIa-30 and VIIa-31.

In the case of VIIa, especially in the experiment using a small substrate, the structures of the 99 and 170 loops clearly seemed to independently affect the $K_m$ and $k_{cat}$ values, respectively. Recently, the hybrid Xa-trypsin protein (swapping the Xa carboxyl-terminal hemisphere for that of trypsin) was designed and analyzed by Hopfner et al. (37). This hybrid protein has the 99 loop derived from Xa and the 170 loop derived from trypsin and shows enhanced amidolytic activities toward chromogenic substrates in the absence of cofactor Va. This enhancement is mainly due to a decrease in the $K_m$ value but not due to an increase in the $k_{cat}$ value. Interestingly, this mutant showed a similar amidolytic activity to that of wild-type IXa in the presence of VIIIa. However, the enhancement of the $k_{cat}/K_m$ value in the 99 loop-modified VIIa (VIIa-30) is mainly due to the decrease in the $K_m$ value (Table I). Although the catalytic domains of various coagulation proteases show similar conformational structures, they display significant differences in specificity and catalytic activity. One of the important factors affecting these variations seems to exist in the loop structures in each of the protease domains. Furthermore, each loop distributed in these coagulation proteases seems to display its own unique function, as described above. Furthermore, from the results of carbamylation, VIIa-39, in both the 99 and 170 loop-replaced mutant, showed no enhancement in the stability of the ion pair between Ile[16] and Asp[194] (data not shown), although the $k_{cat}/K_m$ value of this mutant without sTF reached about half of the value of the

### Table II

| Name | Chromogenic substrates | Amidolytic activities | Enhanced ratios of activity |
|------|------------------------|-----------------------|-----------------------------|
|      | BG/P4                  | P1                    | P2                          | P3                         | VIIa-W | VIIa-30 | VIIa-31 | VIIa-39 | 30/W | 31/W | 39/W |
| Chromozym t-PA | MeSO$_2$ - n-Phe - Gly - Arg | 36.5 | 111.3 | 80.8 | 271.2 | 3.1 | 2.2 | 7.4 |
| S-2288 | d-Ile - Pro - Arg | 23.6 | 77.3 | 229.0 | 342.2 | 3.3 | 9.7 | 14.5 |
| S-2238 | n-Phe - Pip - Arg | 14.0 | 50.6 | 59.5 | 96.7 | 3.6 | 4.3 | 6.9 |
| S-2366 | pyro-Glu - Pro - Arg | 13.6 | 16.7 | 227.3 | 147.4 | 1.2 | 16.7 | 10.8 |
| Chromozym X | MeO-CO - d-Nle - Gly - Arg | 4.0 | 32.6 | 36.5 | 179.7 | 8.1 | 9.1 | 44.7 |
| S-2302 | Nle - Pro - Phe - Arg | 3.8 | 7.3 | 31.6 | 35.0 | 1.9 | 8.2 | 9.1 |
| S-2785 | Z - n-Arg - Gly - Arg | 2.5 | 24.1 | 14.6 | 94.1 | 9.5 | 5.8 | 37.0 |
| S-2444 | pyro-Glu - Glu - Gly - Arg | 1.2 | 5.9 | 16.9 | 61.0 | 5.1 | 14.5 | 52.0 |
| S-2222 | Bz-Ile-Glu(Glu$_{OMe}$) - Gly - Arg | 0.8 | 5.1 | 5.3 | 46.2 | 6.4 | 10.4 | 57.8 |
| S-2403 | pyro-Glu - Glu - Phe - Lys | 0.2 | 0.0 | 5.7 | 1.4 | 0.0 | 25.3 | 6.1 |

* Amino acid residues occupying positions P$_1$ through P$_3$ are shown with blocking groups (BG) occupying the P$_4$ position. Standard three-letter abbreviations for amino acids indicate l-isomers unless otherwise noted. Other abbreviations are: MeSO$_2$, N-methylsulfonyl; Pip, piperidyl; pyro-Glu, pyroglutamyl; MeO-CO, N-methoxycarbonyl; Nle, norleucyl; Z, N-benzyloxy carbonyl; Bz, N-benzoyl; Glu$_{OMe}$, γ-methoxylglutamyl.

* All substrates were employed at 1 mM in 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, 10 mM CaCl$_2$, and 0.1% PEG 8000.
wild-type VIIa-sTF complex in the experiments using a small substrate. On the other hand, recently, several VIIa mutants have been designed and analyzed by Persson et al. (39, 40). One of the mutants had five point mutations with Leu[163]Val, Lys[188]Ala, Val[21]Asp, Glu[154]Val, and Met[156]Gln. This mutant not only showed 11-fold enhancement of the amidolytic activity toward the chromogenic substrate S-2288 as compared with wild-type VIIa but also showed significant enhancement of the stability of the ion pair between Ile[16] and Asp[194], even in the absence of sTF. With stabilization of the ion pair, the amidolytic activity enhancement of this mutant reached about 30% that of the complex between wild-type VIIa and sTF; however, the VIIa-39 mutant (designed here) showed amidolytic activity of about 50% that of the wild-type VIIa-sTF complex without any stabilization of the ion pair. These results suggested the existence of multiple structural mechanisms to regulate the expression of VIIa activity. One of the structural mechanisms is responsible for the stabilization of ion pair formation, as described previously (5, 36, 41, 42). Another structural mechanism is responsible for catalytic efficiency, which could be independent of formation of the ion pair (32, 43, 44).

VIIa is assumed to exist in equilibrium between minor active and dominant zymogen-like inactive conformational states. It is further assumed that preferential binding of TF to the active state leads to a shift in equilibrium (36). We confirmed that sTF dependence on catalytic enhancement of VIIa-31 and VIIa-39 was decreased (Fig. 5) and that the binding mode of VIIa to sTF was affected by the modification of the 170 loop (Fig. 6). These results suggest that the modification of the 170 loop of VIIa not only reduces binding to sTF but also makes association with sTF possible, regardless of whether the molecules are in an active state or inactive state.

In the present study, we prepared two types of VIIa mutants (VIIa-30 and VIIa-39). VIIa-30, 99 loop-modified VIIa, showed similar TF-dependent enhancement of catalytic activity to that of wild type. In either the absence or presence of TF, the catalytic efficiencies of VIIa-30 for small synthetic substrates and natural macromolecular substrate, FX, are higher than those of wild type. On the other hand, VIIa-39, 99 loop- and 170
loop-modified VIIa showed a decrease in TF-dependent enhancement of catalytic activity to that of wild type but has dramatically enhanced catalytic activities for small synthetic substrates and natural macromolecular substrate in the absence of TF. These approaches to develop mutants showing higher catalytic activities than that of wild-type VIIa may be useful in understanding the mechanisms of expressing activity of VIIa and may also serve as a potential treatment of bleeding disorders.

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