Macromolecular Substrate Affinity for the Tissue Factor-Factor VIIa Complex Is Independent of Scissile Bond Docking*

(Received for publication, May 6, 1999, and in revised form, June 9, 1999)

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The upstream coagulation enzymes are homologous trypsin-like serine proteases that typically function in enzyme-cofactor complexes, exemplified by coagulation factor VIIa (VIIa), which is allosterically activated upon binding to its cell surface receptor tissue factor (TF). TF cooperates with VIIa to create a macromolecular recognition surface that serves as an exosite for factor X binding. This study analyzes to what extent scissile bond docking to the catalytic cleft contributes to macromolecular substrate affinity. Mutation of the P1 Arg residue in factor X to Gln prevented activation by the TF-VIIa complex but did not reduce macromolecular substrate affinity for TF-VIIa. Similarly, mutations of the S and S' subsites in the catalytic cleft of the enzyme VIIa failed to reduce affinity for factor X, although the affinity for small chromogenic substrates and the efficiency of factor X scissile bond cleavage were reduced. Thus, docking of the activation peptide bond to the catalytic cleft of this enzyme-cofactor complex does not significantly contribute to affinity for macromolecular substrate. Rather, it appears that the creation of an extended macromolecular substrate recognition surface involving enzyme and cofactor is utilized to generate substrate specificity between the highly homologous, regulatory proteases of the coagulation cascade.

In higher organisms, serine proteases serve highly specialized functions in host defense, wound repair, and differentiation. The chymotrypsin-like subclass of the serine protease family is characterized by a conserved fold of the ~240-residue protease domain, resulting in an optimal positioning of the catalytic triad Ser-195 and His-57 residues in an invariable position (residue positions are based on chymotrypsin numbering; for corresponding VIIa numbering, see Ref. 1). The most important determinant for serine protease specificity is the S1 specificity pocket that accommodates the P1 residue (residue 15 in chymotrypsin numbering) located amino-terminal to the scissile bond (2). The charge and shape properties of this pocket determine the fit of the P1 side chain and the proper presentation of the peptide bond for nucleophilic attack by Ser-195.

Certain subsets of this protease family are evolutionary closely related and constitute functionally cooperating enzyme systems, such as the coagulation cascade (3). In the latter, the proteases share an acidic Asp-189 residue that forms the bottom of the S1 specificity pocket and thus are capable of cleaving very similar substrates that contain an Arg at the P1 position. Despite this identical P1 residue in all zymogen precursors of these enzymes, the coagulation proteases show remarkable macromolecular substrate specificity, allowing for a highly regulated consecutive activation of the individual zymogens in the cascade.

Substrate specificity determinants of these enzymes thus likely involve structural characteristics other than the S1 pocket. A large number of studies, employing either small peptidyl substrate mimics or inhibitors that bind to the active site, have provided evidence that the catalytic clefts of the coagulation proteases have characteristic features that prefer certain residues in more extended positions on both sides of the scissile bond (4–8). Protein engineering has further been employed to demonstrate that determinants in the active site cleft are critical for substrate specificity of coagulation proteases (9–11). However, modifications of small peptide substrates at the P2-P3 or at P' subsite positions typically generate substrate selectivity that differs by less than 10-fold between coagulation enzymes (5, 8, 12). Considering the similarity in sequence at the scissile bond of the coagulation serine protease zymogens, the observed changes in catalytic efficiency for small substrates cannot entirely explain the high degree of selectivity for macromolecular substrate.

Macromolecular substrate activation by coagulation proteases depends on protein cofactor/receptors that localize these enzymes to cell surfaces. In the initiation phase of coagulation, tissue factor (TF), a transmembrane receptor structurally related to the cytokine receptor family, forms a high affinity complex with coagulation factor VIIa (VIIa) (13). One result of TF-VIIa complex formation is allosteric activation of VIIa. VIIa makes an incomplete zymogen to enzyme transition after proteolytic cleavage, and TF binding to the VIIa protease domain likely activates catalytic function by ordering loop segments of the activation domain (14). TF not only influences the catalytic cleft of VIIa but also directly interacts with macromolecular substrate. In particular, Lys residues 165 and 166, located in a membrane proximal position in the carboxyl-terminal module of TF, contribute to protein-protein interactions with the coagulation factor X (X) Glu domain (15, 16). The Glu domain of substrate docks into a larger collision surface constituted by the VIIa Glu domain (17, 18) in complex with the carboxyl-terminal module of TF (19).

Monoclonal antibodies to TF (20) and VIIa (21) have provided further evidence for the location of exosites for macromolecular substrate binding. Notably, these monoclonal antibodies cannot entirely explain the high degree of selectivity for macromolecular substrate.
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ACTIVATION (21, 22). These data are consistent with the hypothesis that proper presentation and activation of natural substrates are critically dependent on binding of the macromolecular substrate with exosite regions involving both cofactor and enzyme. Active site-inhibited thrombin has been shown to act as an efficient competitive inhibitor for macromolecular substrate activation by the prothrombinase complex, leading to the proposal that exosite docking is the major determinant for macromolecular substrate affinity (23). Moreover, a proteolytic fragment of thrombin that corresponds to the carboxyl-terminal half of the protease domain contains the necessary structural elements for macromolecular substrate docking with the prothrombinase complex (24). Because this fragment contains most of the loop segments known as the “activation domain,” which are highly flexible prior to proteolytic zymogen activation or inhibitor modification (25, 26), it remains to be established to what extent the active site blocked thrombin and derived fragments faithfully mimic the cofactor/enzyme docking mode of the zymogen-substrate protease domain.

In order to investigate the role of scissile bond docking within the structural context of the intact zymogen, we mutated the P1 Arg residue in macromolecular substrate X. This mutation of the primary specificity determinant in substrate, as well as of recognition determinants in the catalytic cleft of VIIa that impair the interactions of the scissile bond with the active site of the enzyme, failed to reduce affinity for X, demonstrating that exosite docking is the only critical factor that governs macromolecular substrate affinity for TF-VIIa. By providing direct and independent experimental evidence, these data thus allow generalization of the hypothesis that specificity of the highly homologous, cofactor-dependent upstream coagulation serine proteases is achieved by macromolecular substrate recognition determinants of exosites, rather than sequence specificity of the catalytic cleft.

EXPERIMENTAL PROCEDURES

Proteins—Full-length recombinant human TF was produced from insect cells and reconstituted into 30% phosphatidylserine/70% phosphatidylcholine (TCP/PCPS), or 100% PCPS, as described (27). The soluble extracellular domain of TF, TFΔ1–218, was expressed in Escherichia coli and purified and refolded from inclusion bodies, as described (28). X was purified from plasma, followed by immunoaffinity chromatography on immobilized monoclonal antibody F21–4.2C. Protein with partial degradation of the Gla domain was separated in a barium citrate precipitation step from fully carboxylated VIIa. The display of kinetic parameters for chromogenic substrate Spectrozyme FXa. Initial rate data were fitted to the Michaelis-Menten equation.

The interaction of inactive X mutants with TF-VIIa was analyzed in a competitive inhibition assay with wild-type, plasma-derived X. After forming the TF-VIIa complex on PCPS vesicles under the conditions described above, wild-type X and XArg-15,Ala-195, XGlut-7, and XGlu-8 were treated sequentially, followed by quenching with 30% LPD. The determination of the generation of active Xa with Spectrozyme FXa. Initial rate data were obtained in three independent experiments for wild-type X concentrations of 0.2–10 μM at each of the inhibitor concentrations used. The means of the initial velocity data were fitted to the Michaelis-Menten equation to determine the apparent Km and kcat for X activation. Both mutants showed competitive inhibition of X activation. kcat values were determined from the replots of the Km values versus the competitor concentration (0–400 nM) for each of the mutants.

Amidolytic function of wild-type or mutant VIIa (5 nM) was measured in the presence of increasing concentrations of soluble TFΔ1–218 (0.5–100 nM) with 100 μM chromogenic substrate Chromozym t-PA (Roche Molecular Biochemicals) in Tris-buffered saline, 5 mM CaCl2, pH 8.0. Kinetic parameters for chromogenic substrate hydrolysis were determined at a fixed enzyme (30 nM) and cofactor TFΔ1–218 (120 nM) concentration with varying concentrations of substrate (1.5 mM to 10 μM) under the same buffer conditions.

RESULTS

Determination of Macromolecular Substrate Affinity for TF-VIIa by Competitive Assay—To analyze macromolecular substrate docking with TF-VIIa, we mutated the catalytic triad Ser to Ala to generate a catalytically inactive X molecule. In the first mutant, XArg-15,Ala-195, the scissile bond P1 residue Arg was unchanged. As expected, XArg-15,Ala-195 had no measurable catalytic activity after zymogen activation and thus did not interfere with function. In the second mutant, XGlu-7, Glu-8, lacked amidolytic activity. The scissile bond P1 (2) residue Arg was additionally mutated to Gln in another mutant, XArg-15,Ala-195, to generate a macromolecular substrate mimic that fails to properly dock into the S1 specificity pocket. Both mutants were stably expressed in dhfr-dhfr-deficient Chinese hamster ovary cells, and each protein was purified from serum-free culture supernatants with immobilized anti-X monoclonal antibody P21–4.2C. Protein with partial degradation of the Gla domain was separated in a barium citrate precipitation step from fully γ-carboxylated protein that has a properly folded Gla domain (18). The eluates from the barium citrate precipitate were dialyzed extensively against Tris-buffered saline for storage at 4 °C. Final pass over a benzamidine-Sepharose column equilibrated with Tris-buffered saline was used to eliminate trace contaminants of activated X that may have been generated during production or purification of the recombinant protein. The termini of light and heavy chains of the mutants were confirmed by amino-terminal sequencing, which showed <4% yield for Glu-7 and Glu-8, consistent with complete γ-carboxylation of these Gla peptides.

Functional Assays—Km and kcat for X hydrolysis by TF-VIIa were determined with a fixed concentration (200 μM) of TF/PC or TF/PCPS and excess wild-type or mutant VIIa (1 nM in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4), 5 mM CaCl2, 0.2% bovine serum albumin). After a brief preincubation (5 min) of TF with VIIa to allow for complex formation, X (0.2 nM to 10 mM) was added, and activation was determined in samples quenched with 100 mM EDTA, using the chromogenic substrate Spectrozyme FXa. Initial rate data were fitted to the Michaelis-Menten equation.
X with a $K_\text{m}$ of $34 \pm 3 \text{ nM}$, which is comparable to the $K_\text{m}$ for wild-type X activation under these experimental conditions. Hydrolysis of the peptidyl substrate Chromozym t-PA (0.5 mM) by 30 nM VIIa in complex with 120 nM TF$_{1-218}$ was measured in the presence or absence of 2.5 $\mu$M XArg-15,Ala-195. The rate of hydrolysis in the presence (81 ± 5 relative absorbance/min) or absence (83 ± 3 relative absorbance/min) of the inactive X mutant were indistinguishable, supporting the notion that XArg-15,Ala-195 competes with docking of macromolecular substrate, rather than inhibits scissile bond cleavage in the active site cleft.

**Mutation of the P1 Residue in X Does Not Influence Affinity for Docking with TF-VIIa**—To analyze the importance of the P1 residue for macromolecular substrate affinity, the P1 residue Arg in X was replaced by Gln. Fig. 2 shows the time-dependent activation of catalytically inactive X mutants with either Arg or Gln as the P1 residues. Whereas the activation of XArg-15,Ala-195 approached completion after 15 min, no activation was detectable after 1 h in the case of XGln-15,Ala-195. This demonstrates that mutation of P1 to Gln prevents scissile bond cleavage, which is likely attributable to a failure of the Gln residue to properly bind to the S1 specificity pocket. As found with XArg-15,Ala-195, the P1 Gln mutant XGln-15,Ala-195 (2.5 $\mu$M) failed to inhibit amidolytic function of VIIa (30 nM) in complex with 120 nM TF$_{1-218}$ (83 ± 3 relative absorbance/min without versus 82 ± 3 relative absorbance/min with XGln-15,Ala-195), arguing that this mutant P1 residue does not occupy the S1 subsite without subsequent scissile bond cleavage. XGln-15,Ala-195 was as potent a competitor as XArg-15,Ala-195 for X docking to TF-VIIa. The Lineweaver-Burk plots show competitive inhibition by XGln-15,Ala-195 (Fig. 1), and the calculated $K_I$ of 40 ± 4 mM was indistinguishable from XArg-15,Ala-195. These data demonstrate that the P1 residue in X makes no measurable contribution to the affinity of macromolecular substrate binding to TF-VIIa.

**Mutation of Catalytic Cleft Residues in VIIa**—This conclusion would predict that mutations in the catalytic cleft of VIIa, to which the activation peptide region of macromolecular substrate binds, should not affect the affinity of TF-VIIa for X. To test this hypothesis, two residue positions in the catalytic cleft were selected for replacement with Ala. On the S subsite, Thr-99 makes contacts with the P2 residue, as shown in the crystal structure of the Phe-Phe-Arg-inhibited TF-VIIa complex (19). On the S' subsite, residue Gln-40 was mutated. Although the precise docking of the P' residues of coagulation serine protease substrates is not known, a recent crystal structure of TF-VIIa in complex with the Kunitz-type inhibitor 5L15 shows contacts of Leu-39, adjacent to Gln-40, with the P2' position of the inhibitor (32), indicating perturbation of the proximity of the S2' subsite by the Gln-40 mutation. The mutants were purified to homogeneity and characterized by surface plasmon resonance analysis for binding interactions to validate the overall proper folding of the proteins.

The mutations had little effect on VIIa binding to TF (Table I). In addition, active site modification with the covalent inhibitor Phe-Phe-Arg chloromethyl ketone increased affinity for each of the mutants, as previously found for wild-type VIIa (29). This demonstrates that these mutations in the catalytic cleft do not diminish cofactor binding (TF) or disrupt allosteric changes that link the active site to the cofactor binding site. Using a previously characterized, conformation sensitive monoclonal antibody (F3–3.2A) to the VIIa protease domain exosite that participates in X docking (21), we further analyzed conformational changes in this exosite region of VIIa. VIIA$_{Ala-40}$ and VIIA$_{Ala-99}$ bound to F3–3.2A with the same affinity as wild-type VIIa and displayed a loss of affinity upon active site modification in accordance with that of wild-type (Table I). These data argue that the mutational effect of these two replacements is confined to the active site cleft and does not influence the overall conformation or active site occupancy-related allosteric changes in the VIIa protease domain.

The unaltered affinity for TF is further demonstrated by dose titration with soluble TF$_{1-218}$ in the amidolytic assay shown in Fig. 3. Whereas VIIA$_{Ala-40}$ and VIIA$_{Ala-99}$ hydrolyzed the chromogenic substrate Chromozym t-PA with similar efficiency as wild-type VIIa, a dramatic reduction in amidolytic activity for VIIA$_{Ala-99}$ was observed. Kinetic parameters for cleavage of this substrate were determined at a fixed enzyme concentration, demonstrating that the loss of catalytic function of VIIA$_{Ala-99}$ resulted...
predominantly from a significant decrease in the affinity for the p-nitroanilide substrate (Table II). These results are expected from a modification of the S2 subsite, because the activation of small substrates is highly dependent on the docking to the S1–3 subsite, with little contribution of binding of the p-nitroanilide group to the S’ subsite.

**Effect of Mutations in the Catalytic Cleft on Macromolecular Substrate Affinity**

In this study, site-specific mutagenesis was employed to determine whether binding of the activation peptide region of X to the active site cleft of TF/VIIa plays a significant role in macromolecular substrate affinity. In two independent experimental approaches, we found that scissile bond docking does not measurably contribute to macromolecular substrate affinity of the TF/VIIa complex. First, we generated catalytically inactive X mutants that can be used in a competitive assay with wild-type X. X_{Arg-15,Ala-195} showed pure competitive inhibition of wild-type X activation by TF/VIIa with a K_i that was indistinguishable from the K_m of wild-type X activation. Thus, this mutant truly recapitulates the docking of the macromolecular substrate. Mutation of the P1 Arg residue to Gln on the affinity for small substrate, which solely bind to the S1–3 subsite, with little contribution of binding of the p-nitroanilide group to the S’ subsite.

**TABLE I**

| Binding to TF | \( k_{\text{on}} \) | \( k_{\text{cat}} \) |
|--------------|------------------|------------------|
| Wild-type    | 1.9 ± 0.5 \times 10^5 | 1.6 ± 0.3 \times 10^3 |
| VIIaAla-40   | 3.3 ± 0.6 \times 10^4 | 3.5 ± 0.3 \times 10^2 |
| VIIaAla-99   | 3.2 ± 0.1 \times 10^5 | 1.5 ± 0.2 \times 10^3 |
| FFR-VIIa     | 3.8 ± 0.8 \times 10^3 | 0.5 ± 0.1 \times 10^3 |
| Wild-type    | 3.3 ± 1.4 \times 10^3 | 0.7 ± 0.1 \times 10^3 |
| VIIaAla-40   | 3.7 ± 0.3 \times 10^3 | 0.7 ± 0.1 \times 10^3 |
| VIIaAla-99   | 3.0 ± 0.1 \times 10^3 | 0.6 ± 0.1 \times 10^3 |

**TABLE II**

| Kinetic parameters for chromogenic substrate hydrolysis |
|--------------------------------------------------------|
| \( k_{\text{on}} \) | \( k_{\text{cat}} \) |
| TF_{1-218}^a | Wild-type VIIa | 340 ± 50 \mu M | 145 ± 34 \text{min}^{-1} |
| TF_{1-218} | VIIaAla-40 | 440 ± 20 \mu M | 117 ± 9 \text{min}^{-1} |
| TF_{1-218} | VIIaAla-99 | 1440 ± 170 \mu M | 87 ± 5 \text{min}^{-1} |

**TABLE III**

| Competitive inhibition of X activation by inactive X mutants |
|---------------------------------------------------------------|
| \( X_{\text{Arg-15,Ala-195}} \) | \( X_{\text{Glu-15,Ala-195}} \) |
| Wild-type VIIa | 34 ± 3 | 40 ± 4 |
| VIIaAla-40 | 32 ± 4 | 36 ± 3 |
| VIIaAla-99 | 29 ± 6 | 30 ± 8 |

**Substrate Binding**—The activation of macromolecular substrate X by the two VIIa mutants was analyzed on both charged PCPS and neutral PC vesicles. Mutation of Gln-40 at the S’ subsite did not affect the \( k_{\text{cat}} \), but reduced the \( k_{\text{cat}} \) 2-fold independent of the lipid composition of the vesicles (Table II). These data indicate that S’ subsite docking of the scissile bond makes no contribution to the affinity of macromolecular docking with TF/VIIa, although it is critical for the efficiency of hydrolysis of the peptide bond. This conclusion is further supported by the kinetic parameters for VIIaAla-99 that displayed a greatly reduced affinity for small substrate, which solely bind to the catalytic cleft of VIIa. The \( k_{\text{cat}} \) for X activation was reduced 6-fold upon Thr-99 mutation, but the \( K_m \) was unchanged. Mutations of catalytic clef residues in VIIa thus do not influence macromolecular substrate affinity, although scissile bond-mimicking chromogenic substrates display an increased \( K_m \) for these mutants. These data are consistent with the conclusions drawn from the analysis of mutations of the X P1 position that also did not influence the affinity for TF/VIIa.

Based on these results, one would further predict that the affinities of the inactive X mutants for TF/VIIa are not influenced by the mutations in the catalytic cleft of VIIa. \( X_{\text{Arg-15,Ala-195}} \) and \( X_{\text{Glu-15,Ala-195}} \) were used as competitive inhibitors for wild-type X activation by both VIIa mutants in the presence of TF. The inactive X mutants exhibited pure competitive inhibition with each of the VIIa mutants, as shown for wild-type VIIa in Fig. 1. The \( K_i \) values calculated for these experiments (Table III) demonstrate that \( X_{\text{Arg-15,Ala-195}} \) bound to each of the mutants with the same affinity as to wild-type VIIa in complex with TF. Furthermore, there was no effect of mutation of the P1 Arg residue to Gln on the affinity for the VIIa mutants. These data thus emphasize that neither docking of the scissile bond P1 residue nor interaction at the S or the S’ subsites contributes to X affinity for TF/VIIa, indicating that substrate recognition at exosite regions of cofactor and enzyme is the key determinant for macromolecular substrate binding and specificity.

**DISCUSSION**

In this study, site-specific mutagenesis was employed to determine whether binding of the activation peptide region of X to the active site cleft of TF/VIIa plays a significant role in macromolecular substrate affinity. In two independent experimental approaches, we found that scissile bond docking does not measurably contribute to macromolecular substrate affinity of the TF/VIIa complex. First, we generated catalytically inactive X mutants that can be used in a competitive assay with wild-type X. \( X_{\text{Arg-15,Ala-195}} \) showed pure competitive inhibition of wild-type X activation by TF/VIIa with a \( K_i \) that was indistinguishable from the \( K_m \) of wild-type X activation. Thus, this mutant truly recapitulates the docking of the macromolecular substrate. Mutation of the P1 Arg residue in \( X_{\text{Glu-15,Ala-195}} \) produced a mutant that was resistant to activation by TF/VIIa and that did not inhibit amidolytic function of the complex, indicating that the mutated P1 residue fails to bind productively to the S1 subsite of the enzyme. \( X_{\text{Glu-15,Ala-195}} \) was equally potent as \( X_{\text{Arg-15,Ala-195}} \) as a competitive inhibitor of X activation.
activation by TF-VIIa, demonstrating that P1 docking to the S1 subsite makes no appreciable contributions to macromolecular substrate binding with TF-VIIa in a physiologically assembled ternary complex.

Second, mutations of residues in the S and S′ subsites of the catalytic cleft did not increase the $k_{cat}$ for macromolecular substrate activation. The mutation of the S2 subsite residue significantly reduced the affinity for a small chromogenic p-nitroanilide substrate without major effects on the $k_{cat}$, demonstrating that the catalytic cleft structure is sufficiently perturbed to interfere with normal docking of activation peptide-mimicking pseudosubstrates. However, the mutations in the catalytic cleft of VIIa only reduced the $k_{cat}$ of macromolecular substrate X activation, suggesting that transition state formation and the subsequent acylation/deacylation steps are rate-limiting in protein substrate activation. These data are consistent with a two-step process in which macromolecular substrate docking precedes scissile bond interactions with the catalytic cleft, as suggested for prothrombin activation (23, 24). Other mutations in or near the catalytic cleft of VIIa have previously been shown to have a similar effect on X activation. In particular, Ala replacements for Lys-192 (33), located just above the S1 pocket, and the immediately adjacent Arg-148 in the autolysis loop (27) both reduced the $k_{cat}$ for X activation without influencing the $K_m$. Because Arg-148 is localized in one of the nonconserved loop regions of the serine protease domain that are considered important for substrate specificity (34), the selective effect of the Arg-148 mutation on the $k_{cat}$ may indicate that even these specificity determinants more distant from the scissile bond, but in proximity to the catalytic cleft, are not involved in the initial docking of macromolecular substrate.

Indirect support for a minor importance of scissile bond docking for macromolecular substrate affinity is further provided by the kinetic mechanism of inhibitors of the TF-VIIa complex. Both the nematode inhibitor NAP2e (35) and the natural inhibitor of TF pathway inhibitor (36), are dependent on the product Xα for efficient inhibition of the TF-VIIa complex. Kinetic analysis of the mechanism of inhibition in the latter case clearly demonstrated that the preferred target for inhibition by TFPI is the ternary complex of TF-VIIa-Xα (37), emphasizing that extended macromolecular substrate interactions which may be partially preserved in the product are of sufficient affinity to transiently stabilize the ternary complex. The Gla domain of substrate X is critical for binding to the collision structure of TF and the VIIa Gla domain (15, 16, 18), and mutations in TF that reduce substrate binding also influence the inhibition by the TFPI-Xα complex (38). Furthermore, fusion of the light chain of Xα amino-terminal to TFPI generates an inhibitor chimera with greatly enhanced affinity for TF-VIIa as compared with TFPI alone (39). It thus appears that the light chains of the upstream coagulation proteases evolved to direct the specificity of the interactions between substrates and the cell-surface-associated enzyme cofactor complexes.

The kinetic mechanism of activation of X by TF-VIIa may serve to illustrate a fundamental difference between serine proteases that initiate and propagate enzyme cascades as opposed to effector proteases. Whereas the former typically function in a membrane-localized environment, the latter frequently require diffusion to specific targets and catalyze fluid phase reactions, such as the conversion of fibrinogen to fibrin by thrombin. High affinity for product would significantly impair the catalytic rate of effector proteases. To reduce product inhibition, the cleavage bond vicinity of substrate may have been preferentially evolved as the major determinant for sub-

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