Why Ser and Not Thr Brokers Catalysis in the Trypsin Fold

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ABSTRACT: Although Thr is equally represented as Ser in the human genome and as a nucleophile is as good as Ser, it is never found in the active site of the large family of trypsin-like proteases that utilize the Asp/His/Ser triad. The molecular basis of the preference of Ser over Thr in the trypsin fold was investigated with X-ray structures of the thrombin mutant S195T free and bound to an irreversible active site inhibitor. In the free form, the methyl group of T195 is oriented toward the incoming substrate in a conformation seemingly incompatible with productive binding. In the bound form, the side chain of T195 is reoriented for efficient substrate acylation without causing steric clash within the active site. Rapid kinetics prove that this change is due to selection of an active conformation from a preexisting ensemble of reactive and unreactive rotamers whose relative distribution determines the level of activity of the protease. Consistent with these observations, the S195T substitution is associated with a weak yet finite activity that allows identification of an unanticipated important role for S195 as the end point of allosteric transduction in the trypsin fold. The S195T mutation abrogates the Na⁺-dependent enhancement of catalytic activity in thrombin, activated protein C, and factor Xa and significantly weakens the physiologically important allosteric effects of thrombomodulin on thrombin and of cofactor Va on factor Xa. The evolutionary selection of Ser over Thr in trypsin-like proteases was therefore driven by the need for high catalytic activity and efficient allosteric regulation.

Enzymes utilizing a catalytic Ser as a nucleophile to broker cleavage of peptide bonds are widely distributed in nature and found in all kingdoms of cellular life as well as many viral genomes. A typical genome contains 2–4% of genes encoding proteolytic enzymes, and more than one-third of them are serine proteases. Nucleophilicity of the catalytic Ser is typically achieved through a catalytic triad of Asp/His/Ser residues, commonly termed the charge relay system. The architecture of the triad first emerged almost 50 years ago from the structure of chymotrypsin. The carboxylate of D102 (chymotrypsinogen numbering) accepts a H-bond from the Ne1 atom of H57, which in turn accepts a H-bond at the Ne2 atom from the Oγ atom of S195. The H-bond between D102 and H57 facilitates the abstraction of the proton from S195 and generates a potent nucleophile. The Oγ atom of S195 attacks the carbonyl of the peptide substrate as a result of H57 acting as a general base. The oxyanion tetrahedral intermediate formed in the process is stabilized by the backbone N atoms of G193 and S195, which generate a positively charged pocket within the active site known as the oxyanion hole. H-Bonding interactions in the oxyanion hole contribute 1.5–3.0 kcal/mol to ground and transition state stabilization. Collapse of the tetraheiral intermediate generates the acyl-enzyme, and stabilization of the newly created N-terminus is mediated by H57. A water molecule then displaced the free polypeptide fragment and attacks the acyl-enzyme intermediate. The oxyanion hole stabilizes the second tetrahedral intermediate of the pathway, and collapse of this intermediate liberates a new C-terminus in the substrate.

Combination of the Asp/His/Ser residues of the catalytic triad with the same or opposite handedness exists in at least four main protein folds, i.e., trypsin, subtilisin, prolyl oligopeptidase, and ClpP peptidase, evidence of four different evolutionary origins. Replacement of any member of the triad with other residues in subtilisin and trypsin largely compromises activity, and the effect is nonadditive, indicating that the triad behaves as a highly cooperative unit. The catalytic S195 deserves special attention in the context of evolutionary origins. Ser is unique among amino acids because it is encoded by codons that cannot interconvert by single-nucleotide replacement. Both AGY and TCN codons are documented, indicating that the intermediates Thr and Cys along this transition should also be present in the protease family; however, only a few family members of viral origin use an active site thiol from a Cys residue, and no trypsin-like protease replaces Ser with Thr in the catalytic triad. The absence of Thr as a catalytic residue in trypsin-like proteases is intriguing because Thr is as reactive as a nucleophile as Ser, occurs as frequently in the human genome, and is used as an efficient nucleophile in other enzyme folds. Previous computational and observational studies have speculated that selection of Ser over Thr in the trypsin fold was driven by the steric clash caused by the methyl group within the active site, making Thr incompatible with catalytic activity. Functional support to this claim comes from the observation that the S195T mutant of trypsin is inactive and acquires minimal activity upon replacement of additional residues in the active site that relieve the steric clash. However, no structural

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evidence of the consequences of replacing Ser with Thr in the active site of a trypsin-like protease currently exists, nor is it known whether the S195T substitution has the same functional effect on other members of the trypsin family or causes additional perturbations.

In this study, we report high-resolution X-ray crystal structures of the S195T mutant of the clotting protease thrombin, free and bound to the irreversible inhibitor H-\(\gamma\)-Phe-Pro-Arg-CH\(_2\)Cl (PPACK). The structures document expected and unexpected consequences of replacing Ser with Thr as a nucleophile. Functionally, the mutation reduces but does not abrogate activity and reveals that S195 is needed to broker catalysis and allosteric regulation in the trypsin fold.

\section*{MATERIALS AND METHODS}

\textbf{Reagents.} Wild-type thrombin and mutant S195T were expressed as prethrombin-2 in \textit{Escherichia coli}, refolded, and purified to homogeneity as previously described.\(^{25}\) Protein C was expressed, purified to homogeneity, and activated as described elsewhere.\(^{26,27}\) Factor X cDNA modified to include an epitope for the HPC4 antibody was cloned into a pENTR/D-TOPO expression vector (Life Technologies). The protein was expressed using HEK293 cells, purified using HPC4 affinity chromatography, activated using RVV-X (Haematologic Technologies), and further purified to homogeneity using a Q-Sepharose column.

\textbf{Functional Assays.} The effect of Na\(^+\) on the hydrolysis of the chromogenic substrates H-\(\gamma\)-Phe-Pro-Arg-P-nitroanilide (FPR) for thrombin, pyroGlu-Pro-Arg-P-nitroanilide for activated protein C, or H-\(\gamma\)-Phe-Gly-Arg-P-nitroanilide for factor Xa was studied as detailed elsewhere.\(^{28,29}\) The effect of Na\(^+\) on the hydrolysis of protein C was studied using progress curves of hydrolysis of a substrate specific for thrombin, free and bound to the irreversible inhibitor H-D-Phe-Pro-Arg-\(p\)-nitroanilide (FPR) for thrombin, pyroGlu-Pro-Arg-\(p\)-nitroanilide for activated protein C, or H-\(\gamma\)-Phe-Gly-Arg-\(p\)-nitroanilide for factor Xa as reported elsewhere.\(^{32}\) Stopped-flow fluorescence measurements of binding of FPR to S195T mutant, no appreciable hydrolysis of FPR was detected in <2 weeks in the presence of 8.4 mg/mL protein, 100 mM MES (pH 6.5), 15% PEG6000, 5% MPD, and 2 mM PPACK and were cryoprotected prior to being flash-frozen in a solution of 100 mM MES (pH 6.5), 20% PEG6000, and 40% MPD. X-ray diffraction data were collected with a home source (Rigaku 1.2 kW MMX007 generator with VHF optics) Rigaku Raxis IV detector and were indexed, integrated, and scaled with the HKL2000 software package.\(^{34}\) The structures were determined by molecular replacement using MOLREP from the CCP4 suite\(^{35}\) and Protein Data Bank (PDB) entry 1SHH for thrombin bound to PPACK.\(^{26}\) Refinement and electron density generation were performed with REFMAC5 from the CCP4 suite, and 5% of the reflections were randomly selected as a test set for cross validation. Model building and analysis of the structures were conducted with COOT.\(^{36}\) The structure of S195T bound to PPACK was also subject to a final round of refinement with PHENIX\(^{37}\) and PDB_REDO.\(^{38}\) Ramachandran plots were calculated using PROCHECK.\(^{39}\) Statistics for data collection and refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the PDB (entries 4RKJ for free S195T and 4RKO for S195T bound to PPACK).

\textbf{Analysis of PDB Structures of Trypsin-like Proteases and Zymogens.} Structures with a resolution <2.0 Å from the trypsin Pfam family (PF00089) were obtained from the PDB

| Table 1. Crystallographic Data for Thrombin Mutant S195T |
|-----------------|-----------------|
| buffer          | free            | PPACK-bound     |
| PEG             | 200 mM potassium formate | 100 mM MES (pH 6.5) |
| PDB entry       | 4RKJ            | 4RKO            |
| wavelength (Å)  | 1.54            | 1.54            |
| space group     | P2\(_h\)2\(_h\)2 | P2\(_h\)2\(_h\)2 |
| unit cell dimensions | a = 61.4 Å, b = 91.1 Å, c = 50.5 Å | a = 44.6 Å, b = 73.3 Å, c = 48.7 Å,β = 113.4\(^\circ\) |
| no. of molecules per asymmetric unit | 1 | 1 |
| resolution range (Å) | 40–1.7 | 40–1.85 |
| no. of observations (unique) | 247223 (31895) | 83278 (22779) |
| completeness (%) | 99.8 (98.8) | 92.0 (86.8) |
| R\(_{free}\) (%) | 8.6 (56.1) | 81 (51.8) |
| R/σ(f) | 20.3 (2.9) | 13.4 (1.8) |

Abbreviations: mm, main chain—main chain; ms, main chain—side chain; ss, side chain—side chain.

in <2 weeks in the presence of 8.4 mg/mL protein, 100 mM MES (pH 6.5), 15% PEG6000, 5% MPD, and 2 mM PPACK and were cryoprotected prior to being flash-frozen in a solution of 100 mM MES (pH 6.5), 20% PEG6000, and 40% MPD. X-ray diffraction data were collected with a home source (Rigaku 1.2 kW MMX007 generator with VHF optics) Rigaku Raxis IV detector and were indexed, integrated, and scaled with the HKL2000 software package. The structures were determined by molecular replacement using MOLREP from the CCP4 suite and Protein Data Bank (PDB) entry 1SHH for thrombin bound to PPACK. Refinement and electron density generation were performed with REFMAC5 from the CCP4 suite, and 5% of the reflections were randomly selected as a test set for cross validation. Model building and analysis of the structures were conducted with COOT. The structure of S195T bound to PPACK was also subject to a final round of refinement with PHENIX and PDB_REDO. Ramachandran plots were calculated using PROCHECK. Statistics for data collection and refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the PDB (entries 4RKJ for free S195T and 4RKO for S195T bound to PPACK).

Analysis of PDB Structures of Trypsin-like Proteases and Zymogens. Structures with a resolution <2.0 Å from the trypsin Pfam family (PF00089) were obtained from the PDB
and analyzed. The structurally analogous residues corresponding to H57 and S195 were determined by superimpositioning each structure against a reference molecule (PDB entry 1SHH) using FAST. Structures with mutations at either H57 or S195 were excluded from analysis, resulting in a total of 565 independent chains. No structure of a trypsin-like protease carrying T195 was detected in the PDB. The structures were then assessed to determine if the active site was occupied either by residues from other chains (including symmetry mates) or by a ligand or other heteroatom resulting in 175 chains in this group. For all 565 chains, the dihedral angle (C=Ca−Cβ−Oγ) of the residue corresponding to S195 was calculated and adjusted to the range of 0°–360°. The H-bonding distance between S195 and H57 was measured from the Ne2 atom of H57 to the Oγ atom of S195.

**RESULTS**

The consequences of replacing Ser with Thr as an active site nucleophile in the trypsin fold are revealed directly by the crystal structures of the S195T mutant of the clotting protease thrombin free and bound to PPACK. The structure in the free form, determined at 1.7 Å resolution (Table 1), shows the active site in the open E conformation, with a Ca−Ca distance between the two conserved residues G193 and G216 of 10.3 Å and the side chain of W215 pointing away from the active site entrance (Figure 1A). The rmsd between the structure of S195T free and the E form of the wild type is only 0.28 Å. The substituted T195 is clearly visible in the density map and has no influence on critical interactions within the active site, such as the H-bond between the catalytic D102 and H57 or the ion pair between the N-terminal residue I16 and H57 needed to correctly fold the oxyanion hole upon thezymogen to protease transition. However, the methyl group of T195 points to the solvent and toward an incoming substrate with which it would be in direct steric clash and sits in a position typically occupied by the Oγ atom of S195. The Oγ atom of T195 points in the opposite direction relative to the methyl group, toward the neighbor C42−C58 disulfide bond that remains 3.1 Å away, but makes a strong H-bonding interaction (2.9 Å) with the Ne2 atom of H57. The position of the H atom on the Ne2 atom of H57 estimated after optimization with PROPKA at pH 7.0 is within 1.95 Å of the Oγ atom of T195 at an H-bond acceptor angle of 91.5°, consistent with the geometry expected of a strong H-bond. The molecular defect of T195 as a nucleophile in the trypsin fold is not due to a lack of H-bonding interaction with H57, but rather to an “unreactive” rotamer that positions the methyl group in direct clash with the incoming substrate.

Previous studies also speculated that activity in the S195T mutant would require a rotation of the T195 side chain impeded by the ensuing steric clash of the methyl group with the C42−C58 disulfide bond or the catalytic H57. However, removal of the C42−C58 disulfide bond with Ala and Val substitutions confers on the S195T mutant of trypsin a significant level of activity only toward ester substrates. The mobility of T195 within the active site is directly documented by the structure of the S195T mutant bound to the irreversible inhibitor PPACK (Figure 1B) determined at 1.85 Å resolution (Table 1). A rotation of the side chain of T195 brings the Oγ atom into covalent interaction with PPACK as seen in the wild type and repositions the methyl group to the back of the active site cavity next to the C42−C58 disulfide bond, but without causing any appreciable steric clash. Overall, the structure is practically identical (rmsd of 0.25 Å) to that of wild-type bound to PPACK in the Na+ bound form. The inhibitor binds to the active site with all expected H-bonding interactions with D189, the oxyanion hole, and the backbone O and N atoms of G216, as well as with a strong edge-to-face interaction with W215 (Figure 1B). Comparison of the free (Figure 1A) and PPACK-bound structures (Figure 1C) proves that the side chain of T195 may assume at least two different orientations within the active site without encroaching on the catalytic H57 or the C42−C58 disulfide bond. This observation bears on the role of S195 as a nucleophile.

**Figure 1.** Active site conformations of the thrombin mutant S195T free (A) or bound to PPACK (B and C) as determined by X-ray crystallography (Table 1). The side chain of T195 positions the methyl group toward an incoming substrate and the Oγ atom in the opposite direction but 3.1 Å from the C42−C58 disulfide bond. This defective conformation of the nucleophile is corrected upon binding of PPACK (B, yellow sticks) that makes all the expected contacts with residues within the active site. The PPACK-bound conformation (C, PPACK removed for the sake of clarity) has an orientation of the Oγ atom of T195 compatible with substrate acylation. Shown are relevant H-bonding interactions. PPACK makes the expected contacts with the N atoms of T195 and G193 in the oxyanion hole, the N and O atoms of G216, and the carboxylate of D189, as well as a strong edge-to-face interaction with W215 in the aryl binding site. The presence of T195 does not alter the critical H-bonding interactions between D102 and H57 or the ion pair between D194 and the N-terminal I16 that stabilizes the active site and oxyanion hole. There is no steric clash in the two orientations of T195 with residues within the active site. The distance of the methyl group from H57 is 4.1 Å in the free form (A) and 3.2 Å in the PPACK-bound form (C). Electron density (green mesh) is a 2Fo−Fc map contoured at 1.0σ.
Experimental conditions were 0.1% PEG8000, 400 mM ChCl, and 50 mM Tris (pH 8.0) at 15 °C in the presence of ligands within the active site. Features of the Gaussian distribution with a resolution (<2.0 Å) structures of trypsin-like proteases and zymogens deposited in the PDB. The H-bonding distance between S195 and H57 features a Gaussian distribution with a μ value of 3.1 Å and a σ value of 0.3 Å barely affected by the presence of ligands in the active site (μ = 3.0 Å, and σ = 0.3 Å). The S195 residue adopts a Gaussian distribution of dihedral angles with a μ of 156° and a σ of 37°, which is affected minimally by the presence of ligands within the active site (μ = 148°, and σ = 36°). H-bonding distances and dihedral angles are also reported for the few zymogen structures documented in the PDB for the sake of completeness. The sample is, however, too small for statistical comparisons. (C) There is no correlation (r ≤ 0.51) between the dihedral angle of S195 and its H-bonding distance from H57 in all three data sets.

There is observational evidence that S195 itself is highly mobile within the active site. Analysis of 565 high-resolution (<2.0 Å) structures currently deposited in the PDB documents a Gaussian distribution for both the H-bonding distance between the Oγ atom of S195 and the Nε2 atom of the catalytic HS7 (Figure 2A) and the dihedral Cα−Ca−Cβ−Oy angle of S195 (Figure 2B). There is no significant correlation (r = 0.51) between the two quantities (Figure 2C), indicating that a stable H-bond between S195 and H57 can be established in multiple orientations of S195 within the active site. The dihedral angle of 305° observed with T195 in the free form (Figure 2B) lies outside of the Gaussian distribution of S195 and is clearly incompatible with a productive conformation of the nucleophile (Figure 1A), yet the H-bonding interaction with H57 remains strong. The relatively large set of dihedral angle and H-bonding arrangements available to S195 should be compatible with multiple levels of substrate binding and acylation, creating the potential for fine-tuning catalytic activity with minimal perturbation of the structure.

The structure of the S195T mutant reported here is the first and only structure of a trypsin-like protease carrying Thr as a nucleophile, which makes it difficult to extend the observations made on S195 (Figure 2). If the conformation documented in the crystal structure of the S195T mutant in the free form is a genuine representation of an energetically stable rotamer of the nucleophile, it may be expected that the Gaussian distribution accessible to T195 is shifted toward rotamers with higher values of the dihedral angle (Figure 2B) that are unfavorable to substrate binding and catalysis. Importantly, binding of PPACK corrects this defect and shifts the dihedral angle toward a value (160°) that is closer to the mean (148°) of the S195 distribution (Figure 2B) without causing any significant steric clash within the active site. A facile conclusion suggested by the crystal structures is that binding of substrate induces a shift in the rotamer of T195 to facilitate acylation, but rapid kinetics reveal a different scenario. The chromogenic substrate FPR is the cleavable analogue of PPACK and binds to the S195T mutant on the millisecond time scale over which no significant cleavage by the enzyme takes place (Figure 3A). The single-exponential relaxation associated with the binding interaction (kobs) is independent of FPR concentration (Figure 3B). Induced fit requires the value of kobs to increase hyperbolically...
The energetic cost of rotating ± T195 within the active site for optimal acylation and binding is significant and the Na⁺ effect is practically abrogated (for activated protein C, \( s_0 = 0.0002 \pm 0.0001 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 0.36 \pm 0.02 \text{mM}^{-1} \text{s}^{-1} \)). The \( K_d \) value of 2.7 ± 0.1 nM for binding of thrombomodulin to the wild type is practically identical to the \( K_d \) value of 2.6 ± 0.1 nM for the S195T mutant. Experimental conditions were 0.1% PEG8000, 145 mM NaCl, 5 mM CaCl₂, and 5 mM Tris (pH 7.4) at 37 °C.

Figure 4. (A) Effect of Na⁺ on the value of \( s = k_{cat}/K_m \) for the hydrolysis of FPR by wild-type thrombin (red) and S195T (blue). Under the effect of a cofactor, the value of \( s \) increases according to the linkage expression \( s = (s_0 K_d + s_1 x)/(K_d + x) \), where \( s_0 \) and \( s_1 \) are the asymptotic values of \( s \) at \( x = 0 \) and \( x \rightarrow \infty \), respectively, and \( K_d \) is the apparent equilibrium dissociation constant for the cofactor. The values of \( s \) are expressed in units of \( s_0 \) to facilitate comparison. In the case of the wild type, the value of \( s \) increases from an \( s_0 \) of 3.8 ± 0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 92 ± 2 \( \mu \text{M}^{-1} \text{s}^{-1} \). In the case of S195T, the activity decreases drastically and the Na⁺ effect is practically abrogated (\( s_0 = 1.8 \pm 0.1 \text{mM}^{-1} \text{s}^{-1} \), and \( s_1 = 2.3 \pm 0.1 \text{mM}^{-1} \text{s}^{-1} \)). Experimental conditions were 0.1% PEG8000 and 5 mM Tris (pH 8.0) at 25 °C. (B) Na⁺ binding curve for the wild type (red) and S195T (blue) obtained by titration of the intrinsic fluorescence of the protein. The total change in fluorescence was 11% for the wild type and 13% for S195T. Mutation of S195 has little effect on the value of the equilibrium dissociation constant \( K_d \) that changes from 8.2 ± 0.8 mM in the wild type to 62 ± 0.2 mM in the mutant. Experimental conditions were 0.1% PEG8000 and 50 mM Tris (pH 8.0) at 15 °C. (C) Effect of thrombomodulin on the value of \( s = k_{cat}/K_m \) for the hydrolysis of protein C by wild-type thrombin (red) and S195T (blue). The values of \( s \) are expressed in units of \( s_0 \) as in panel A to facilitate comparison. In the case of the wild type (red), the value of \( s \) increases 2100-fold from an \( s_0 \) of 0.15 ± 0.01 \( \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 310 ± 10 \( \mu \text{M}^{-1} \text{s}^{-1} \). In the case of S195T (blue), the activity decreases drastically and the effect of thrombomodulin is significantly reduced (\( s_0 = 0.0002 \pm 0.0001 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 0.36 \pm 0.02 \text{mM}^{-1} \text{s}^{-1} \)). The \( K_d \) value of 2.1 ± 0.1 \( \mu \text{M} \) for binding of thrombomodulin to the wild type is significantly reduced from \( s_0 = 0.02 \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 0.11 ± 0.01 \( \mu \text{M}^{-1} \text{s}^{-1} \) for activated protein C (A) and from \( s_0 \) of 1.4 ± 0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 28 ± 1 \( \mu \text{M}^{-1} \text{s}^{-1} \) for factor Xa (B). In the case of the S195T mutant, activity decreases significantly and the Na⁺ effect is practically abrogated (\( s_0 = 0.11 \pm 0.01 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 0.20 \pm 0.01 \text{mM}^{-1} \text{s}^{-1} \); for factor Xa, \( s_0 = 18 \pm 1 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 38 \pm 1 \text{mM}^{-1} \text{s}^{-1} \)). Experimental conditions were (A) 0.1% PEG8000, 5 mM EDTA, and 5 mM Tris (pH 8.0) at 25 °C and (B) 0.1% PEG8000, 5 mM EDTA, and 50 mM HEPES (pH 8.0) at 25 °C.

Figure 5. Effect of Na⁺ on the value of \( s = k_{cat}/K_m \) for the hydrolysis of a chromogenic substrate by the wild type (red) and mutant S195T (blue) of activated protein C (A) and clotting factor Xa (B). The values of \( s \) are expressed in units of \( s_0 \) as in Figure 4 to facilitate comparison. As observed for thrombin (Figure 4A), the value of \( s \) in the wild type increases from an \( s_0 \) of 21 ± 0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 69 ± 2 \( \mu \text{M}^{-1} \text{s}^{-1} \) for activated protein C (A) and from \( s_0 \) of 1.4 ± 0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) to an \( s_1 \) of 28 ± 1 \( \mu \text{M}^{-1} \text{s}^{-1} \) for factor Xa (B). In the case of the S195T mutant, activity decreases significantly and the Na⁺ effect is practically abrogated (for activated protein C, \( s_0 = 0.11 \pm 0.01 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 0.20 \pm 0.01 \text{mM}^{-1} \text{s}^{-1} \); for factor Xa, \( s_0 = 18 \pm 1 \text{mM}^{-1} \text{s}^{-1} \) and \( s_1 = 38 \pm 1 \text{mM}^{-1} \text{s}^{-1} \)). The \( K_d \) value of 2.7 ± 0.1 nM for the S195T mutant. Experimental conditions were 0.1% PEG8000, 145 mM NaCl, 5 mM CaCl₂, and 5 mM Tris (pH 7.4) at 37 °C.

with ligand concentration, while selection from a preexisting ensemble is more versatile and gives rise to values of \( k_{cat} \) that increase with, decrease with, or are independent of ligand concentration. The data in Figure 3 provide unequivocal evidence that binding of FPR to the S195T mutant of thrombin occurs by selecting a productive conformation of the nucleophile from a preexisting ensemble of reactive and unreactive rotamers.

Consistent with the observations from X-ray structural biology and rapid kinetics, the S195T substitution brings about significant functional perturbations of thrombin and a >2000-fold decrease in the value of \( k_{cat}/K_m \) relative to that of the wild type because of a 60-fold increase in \( K_m \) and a 40-fold decrease in \( k_{cat} \) with no evidence of changes in the underlying kinetic mechanism of hydrolysis. The energetic cost of rotating T195 within the active site for optimal acylation and binding is reflected in the perturbed values of \( K_m \) and \( k_{cat} \). That is consistent with the properties of other site-specific perturbations of the enzyme affecting residues in the primary specificity site or the oxyanion hole for which naturally occurring mutations exist in the trypsin family. Absolute selection of Ser over Thr as the nucleophile in the trypsin fold was therefore driven by additional needs. Indeed, an important and completely unanticipated consequence of the S195T substitution is the resulting perturbation of allosterys effects. Trypsin-like proteases carrying Tyr or Phe at position 225 are endowed with Na⁺-dependent enhancement of catalytic activity. The Na⁺ binding site is located >15 Å from residues of the catalytic triad and nestles between the 186- and 220-loops that also control the primary specificity of the enzyme. The Na⁺ effect is allosteric and influences the catalytic activity of highly specific proteases involved in blood
coagulation and the complement system. Binding of Na’ increases $k_{cat}/K_m$ for the hydrolysis of FPR by thrombin nearly 25-fold and is abrogated by the S195T mutation (Figure 4A). The $K_e$ for the thrombin–Na’ interaction is unaffected by the S195T substitution (Figure 4B), proving that S195 is involved exclusively in the allosteric transduction of binding of Na’ into enhanced catalytic activity. This long-range communication is likely mediated by residues D221 in the Na+ loop and N143 next to the oxyanion hole, as identified by previous mutagenesis studies.28,35 The S195T mutation also perturbs a physiologically important allosteric pathway. Binding of thrombomodulin to exosite I located >15 Å from the active site53,54 enhances activity toward anticoagulant protein C >2,000-fold.55 The effect is significantly reduced by the S195T mutation without appreciable perturbation of the binding affinity of the cofactor (Figure 4C). The intriguing functional consequences of the S195T mutation extend to other members of the trypsin fold. The S195T mutation abrogates the Na’ effect in activated protein C and factor Xa (Figure S) and significantly reduces the physiologically important allosteric effect of cofactor Va on factor Xa56 from 3200- to 200-fold (Table 2). Altogether, these functional data prove that S195 not only ensures optimal catalytic activity but also functions as the end point of allosteric transduction in the trypsin fold. In the case of small cofactors like Na’, perturbation of S195 is sufficient to abrogate the allosteric effect. In the case of macromolecular cofactors like thrombomodulin and factor Va, the residual enhancement of catalytic activity (Figure 4C and Table 2) is ascribed to the additional effect that these cofactors exert on the substrates protein C27,57 and prothrombin,58 respectively.

### DISCUSSION

Previous studies have addressed the important question of why Ser and not Thr brokers catalysis in trypsin-like proteases.24 In addition to being an intermediate between the alternative AGY and TCN codons for Ser in the genetic code, two choices of rotamers in the presence of Thr (Figure 2A) and toward unreactive rotamers in the presence of Thr (Figure 2A), thereby explaining the significant loss of catalytic activity in the S195T substitution.

There is an additional benefit in using Ser and not Thr as nucleophile in the trypsin fold. Several proteases carrying S195 such as tissue-type plasminogen activator59 and clotting factors VIIA60 and Xa56 feature extremely low activity toward their physiological targets in the absence of cofactors, namely, fibrin, tissue factor, and cofactor Va, respectively. Thrombin itself has miniscule activity toward anticoagulant protein C in the absence of thrombomodulin40 and significantly reduced activity toward synthetic and procoagulant substrates in the absence of Na’.62 The observation that the S195T mutation abrogates the allosteric effect of Na’ in thrombin, activated protein C, and factor Xa (Figures 4A and 5) and significantly reduces the allosteric effect of thrombomodulin in thrombin (Figure 4C) and of cofactor Va in factor Xa (Table 2) proves that the absolute selection of Ser over Thr also ensures optimal allosteric regulation in the trypsin fold.

The wide range of rotamers accessible to S195 (Figure 2B) suggests that the level of activity of the protease may be fine-tuned by small rearrangements of the nucleophile within the active site. This energetically favorable landscape is not

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**Table 2. Kinetic Parameters for Activation of Prothrombin by Wild-Type Factor Xa and Mutant S195T**

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<th>with cofactor Va</th>
<th>without cofactor Va</th>
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<td>$k_{cat}/K_m$ ($\mu$M$^{-1}$ s$^{-1}$)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
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<tr>
<td><strong>wt</strong></td>
<td>0.12 ± 0.01</td>
<td>0.022 ± 0.002</td>
</tr>
<tr>
<td>S195T</td>
<td>(0.31 ± 0.03) × 10$^{-3}$</td>
<td>(0.049 ± 0.003) × 10$^{-3}$</td>
</tr>
</tbody>
</table>

“From ref 32. Experimental conditions were 25 μM phospholipids, 150 mM NaCl, 5 mM CaCl$_2$, 0.1% PEG8000, and 20 mM Tris (pH 7.4) at 25 °C, with or without 40 nM cofactor Va. The mutant S195T concentration was 1 nM (without cofactor Va) or 40 pM (with cofactor Va).
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accessible to Thr and compromises allosteric transduction in the trypsin fold. A paradigm shift emerges in which a rich landscape of catalytic regimens is generated by little perturbation of structure, possibly through changes in protein entropy and dynamics. Consequently, the action of allosteric effectors must be precisely exercised through structural changes that culminate in optimization of the rotamer of S195. The subtlety underscoring the role of S195 structural changes that culminate in optimization of the active site of thrombin as the end point of allosteric transduction in the trypsin fold explains why the architecture of the active site of thrombin changes little upon binding of Na+, or thrombomodulin, and why it is difficult to engineer Na+-dependent allostery in proteases devoid of Na+ binding or to reproduce the allosteric effect of thrombomodulin on thrombin and of cofactor Va in the prothrombinase complex.

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