Conversion of Trypsin into a Na^+-Activated Enzyme[†,‡]

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ABSTRACT: Serine proteases of the chymotrypsin family show a dichotomous amino acid distribution for residue 225. Enzymes carrying Tyr at position 225 are activated by Na^+, whereas those carrying Pro are devoid of Na^+ binding and activation. Previous studies have demonstrated that the Y225P conversion is sufficient to abrogate Na^+ activation in several enzymes. However, the reverse substitution P225Y is necessary but not sufficient to introduce Na^+ binding and activation. Here we report that *Streptomyces griseus* trypsin, carrying Pro-225, can be engineered into a Na^+-activated enzyme by replacing residues in the 170, 186, and 220 loops to those of coagulation factor Xa. The findings represent the first instance of an engineered Na^+-activated enzyme and a proof of principle that should enable the design of other proteases with enhanced catalytic activity and allosteric regulation mediated by monovalent cation binding.

Serine proteases of the S1 family are involved in a number of physiologically important functions, including digestion, matrix remodeling, fibrinolysis, complement activation, and blood coagulation (*1*). Determinants of substrate recognition and specificity in this large family of enzymes have been studied in detail (*2*−*4*). A notable feature has emerged among members of the complement system and blood coagulation: some of these proteases belong to the large family of enzymes activated by monovalent cations (*5*, *6*), and their catalytic activity is enhanced specifically and allosterically by Na^+ binding (*7*−*12*). As the majority of serine proteases are devoid of Na^+ binding and activation, significant efforts have been devoted to the elucidation of the determinants of Na^+ binding and allosteric transduction with the objective of introducing such property into any member of the family. Enhancement of catalytic activity may prove to be beneficial in many cases [for example, the lysis of a fibrin clot by tissue-type plasminogen activator (*13*)], and may offer potential benefits to biotechnological and medical applications.

Soon after the structural identification of the first Na^+-binding site in a serine protease (*14*), it became obvious that the single most important difference between proteases activated by Na^+ and those devoid of such property was due to the nature of residue 225 (chymotrypsinogen numbering) (*12*, *15*). Residue 225 is either a Pro or Tyr in the vast majority of serine proteases (*1*, *12*), and such dichotomous distribution is all the more remarkable because the amino acid codons of Pro and Tyr cannot interconvert by a single nucleotide substitution. Pro is found in more ancestral proteases and practically all chymotrypsin-like proteases. Tyr is found in more modern lineages, such as the complement system and the vitamin K-dependent proteases of blood coagulation. Tyr-225 ensures an optimal architecture for Na^+ binding (*15*) and is part of a conserved KYG motif that shares striking similarities with the GYG sequence of the selectivity filter in the K^+ channel (*6*, *16*). The presence of Pro-225 in proteases such as trypsin and chymotrypsin forces the carbonyl oxygen atom of residue 224, one of the Na^+ ligands, in a direction that is incompatible with Na^+ coordination. The Y225P replacement in thrombin (*12*, *17*), activated protein C (*18*), and coagulation factors VIIa (*19*) and Xa (*20*) abrogates Na^+ activation. However, the reverse substitution P225Y in tissue-type plasminogen activator does not result in Na^+ binding or activation (*13*). Hence, Na^+ binding and activation depend on the nature of residue 225 as a necessary but not sufficient condition, just as the nature of residue 189 in the primary specificity pocket is a necessary but not sufficient condition for enzyme specificity (*21*). Indeed, recent mutagenesis studies on thrombin have shown that residues located up to 15 Å away from the Na^+ site influence the binding of Na^+, with residues in the 170, 186, and 220 loops playing a dominant role (*22*). Similar conclusions have been drawn for coagulation factor Xa (*23*). In this paper we report how mutagenesis of these three loops is necessary and sufficient to convert *Streptomyces griseus* trypsin (SGT) into a Na^+-activated enzyme.

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Growth Conditions. Unless stated otherwise, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *Escherichia coli* was grown using standard methods (*24*). *Bacillus subtilis* strain WB700 was grown in superrich medium or on tryptose

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[‡] The atomic coordinates and structure factors (PDB ID 2FMJ) have been deposited in the Protein Data Bank.

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Table 1: Mutants of SGT Described in This Study[a]

construct	mutations
220	Y217E/P222K/Y224K/P225Y
186 + 220	G186K/G187Q/V188E
	Y217E/P222K/Y224K/P225Y
Y172S + 220	Y172S
	Y217E/P222K/Y224K/P225Y
Y172S + 186 + 220	Y172S
	G186K/G187Q/V188E
	Y217E/P222K/Y224K/P225Y
170 + 186 + 220	A171S/Y172S/G173S/N174F/E175I/E180M
	G186K/G187Q/V188E
	Y217E/P222K/Y224K/P225Y

[a] Extensive mutagenesis was applied to introduce and subsequently improve Na^+ activation in SGT. Mutations were introduced to mimic residues found at homologous positions in FXa. Deletion of residue Pro-185 was present in all constructs to ensure proper length of the 186 loop. Mutagenesis of residue 172 to Asn, Asp, His, or Met in the Y172S + 186 + 220 loop mutant was also examined.

blood agar base (BD Diagnostic Systems, Franklin Lakes, NJ) at 37 °C. For *B. subtilis* carrying plasmid pWB980, kanamycin was added to a final concentration of 10 μg mL^{-1} in liquid and solid media. Oligonucleotides for mutagenesis were synthesized by Integrated DNA Technologies (Skokie, IL). Site-directed mutagenesis using the QuikChange method was applied to the SGT gene in the pBluescript *E. coli* cloning vector (Stratagene, La Jolla, CA) and subcloned into the pWB980 using T4 DNA ligase (New England Biolabs, Ipswich, MA). Transformation of *B. subtilis* WB700 was by the method of Spizizen (*25*). Table 1 lists the mutants constructed and characterized in this study. *B. subtilis* clones were validated by DNA sequence analysis of purified plasmid DNA.

Protein Expression and Purification. Proteases were purified from the clarified supernatant of 3 L cultures of recombinant *B. subtilis* after 20 h of growth or until maximum proteolytic activity was observed. The purification protocol applied is a simplified version of the previously reported scheme and incorporates soybean trypsin inhibitor agarose affinity chromatography (*26*). Choline chloride (ChCl)[1] was substituted for NaCl to eliminate Na^+ from the final product. The ionic strength of culture supernatants was increased by 350 mM with ChCl, and the resulting media were applied directly to soybean trypsin inhibitor agarose (10 × 2.5 cm). Washing with 20 column volumes of 50 mM Tris and 500 mM ChCl, pH 7.5, removed the majority of the impurities from the supernatant. Protease was eluted from the affinity column with 10 mM glycine and 20 mM $CaCl_2$, pH 2.0, and concentrated in a Centricon centrifugal concentrator (10000 MW cutoff). Homogeneous protein resulted from a final purification step using Sephadex G-75 (50 × 1.5 cm) (50 mM Tris, 150 mM ChCl). Dialysis against 10 mM calcium acetate, pH 6.1, was followed by concentration in a fresh centrifugal concentrator. Protein purity was >98% by active site titration using PPACK. All mutants were stable for months at 4 °C with no signs of autolytic degradation.

Kinetic Analysis. Hydrolysis of Spectrozyme FXa (American Diagnostica, Stamford, CT) was performed in 50 mM Tris and 2 mM $CaCl_2$, pH 8.0 at 25 °C, in the presence of differing monovalent cations as chloride salts in various concentrations indicated in the figure legends. Analysis of the progress curves for the release of *p*-nitroaniline as a function of substrate concentration was used to estimate the values for k_{cat} and K_M and accounted for product inhibition which was determined to be negligible for all mutants (*27*). Separate values for k_{cat} and K_M could not be obtained for 186 + 220 loop mutant enzymes bearing substitutions at Tyr-172 due to K_M values that exceeded 600 μM. Data were fit to the linkage expression (*28*)

$$s = \frac{s_0 + s_1 K_A x}{1 + K_A x} \quad (1)$$

where $x = [Na^+]$, s_0 and s_1 are the values of $s = k_{cat}/K_M$ for $x = 0$ and $x = \infty$, and $K_A = 1/K_d$ is the equilibrium association constant for Na^+ binding to the free enzyme.

Crystallization and Structure Determination. Hanging-drop crystallization was used to prepare diffraction quality crystals of the 220-loop mutant of SGT. Protein (2 μL of 10 mg/mL in 10 mM calcium acetate, pH 6.1, with 0 or 200 mM LiCl, NaCl, KCl, or RbCl) was mixed with an equal volume of reservoir buffer containing 1.8 M ammonium sulfate containing an equal concentration of the monovalent cation chloride salt as required. Crystals could not be obtained at higher concentrations of salts tested. Diffraction quality crystals appeared within 2 weeks at 25 °C. Data were collected at 100 K (Oxford Cryostream) with an RAXIS-IV detector mounted on a Rigaku RU-200 X-ray generator (50 kV, 100 mA) with Osmic focusing mirrors. Crystals were soaked briefly in artificial mother liquor containing 20% glycerol prior to data collection. Cryoprotectant solution contained 200 mM LiCl, NaCl, KCl, or RbCl where required to maintain the presence of the ion. Data were processed using the HKL package and refined using CNS version 1.1 in combination with XtalView (*29*−*31*). The previously reported recombinant wild-type SGT structure (PDB ID 1OS8) was used as a model for rigid body refinement (*26*). The final model of the 220-loop mutant presents excellent structural parameters and stereochemistry, as summarized in Table 2. The atomic coordinates and structure factors (PDB ID 2FMJ) have been deposited in the Protein Data Bank.

RESULTS AND DISCUSSION

Experimental Strategy. S. griseus trypsin (SGT) expressed from *B. subtilis* provides a useful platform for engineering novel catalytic properties into a trypsin-like enzyme. SGT is one of only a few trypsin-like enzymes (S1 family of peptidases) of bacterial origin (*32*). Previously, we reported that recombinant SGT could be produced in *B. subtilis* and engineered to possess enhanced specificity for Arg side chains at the P1 position (*26*). To further understand catalysis in trypsin-like enzymes, we have begun to reverse-engineer complex properties observed in eukaryotic proteases into SGT. Blood coagulation proteases are highly evolved to possess activity and selectivity. Activation of clotting proteases by Na^+ is tightly interwoven with activity and substrate selectivity (*17*, *33*). Introduction of Na^+-dependent activation similar to coagulation proteases in a digestive protease has not been achieved previously despite significant effort. In the present article, we describe how Na^+ binding and

[1] Abbreviations: aPC, activated protein C; ChCl, choline chloride; FXa, coagulation factor Xa; SGT, *Streptomyces griseus* trypsin; t-PA, tissue-type plasminogen activator.

Table 2: Data Collection and Refinement Statistics for the Crystal Structure of the 220-Loop Mutant of SGT[a]

data collection	
resolution (Å)	1.66 (1.65−1.75)
unique observations	22153
completeness (%)	97.4 (77.2)
average redundancy	5.3 (3.6)
$I/\sigma I$	37.5 (8.0)
R_{merge} (%)	3.2 (15.5)
refinement statistics	
space group	$P6_5$
cell dimensions (*a*, *b*, *c*) (Å)	58.319, 58.319, 100.337
cell angles (α, β, γ) (deg)	90.0, 90.0, 120.0
molecules per asymmetric unit	1
R_{cryst}	0.157
R_{free}	0.189
protein atoms	1616
solvent atoms per asu	272
average *B*-factor for protein (Å^2)	16.09
average *B*-factor for water (Å^2)	28.54
bond length deviations (Å)	0.015
bond angle deviations (deg)	1.7
Ramachandran plot	
most favored (%)	87.5
additionally allowed (%)	12.5
disallowed (%)	0.0

[a] PDB ID 2FMJ. Statistics for the highest resolution shell are given in parentheses.

catalytic rate enhancement can be attained in a trypsin-like protease when a buried hydrophobic residue (Tyr-172) not in direct contact with Na^+ or substrate is substituted by other less bulky side chains in addition to a particular composition of the 186 and 220 loops of the enzyme. Blood coagulation factor Xa (FXa) was chosen as a template to mimic Na^+-dependent activation as the critical Na^+-binding loops resemble SGT in both length and composition. Na^+-dependent activation similar to FXa was successfully introduced into SGT after several cycles of sequential mutagenesis and kinetic analysis.

Residue 225 is Pro in the majority of trypsin-like enzymes and Tyr in Na^+-activated proteases (*1*, *12*). A critical role for Tyr-225 is demonstrated by the Y225P mutation which abolishes Na^+ activation in thrombin (*17*), activated protein C (*18*), and coagulation factors VIIa (*19*) and Xa (*20*). SGT bearing the double mutation P225Y and ΔP185 was initially constructed and determined to be devoid of Na^+ activation. Deletion of Pro-185 generated a 186 loop of equal length to FXa. The length of the 186 loop has been shown to be critical in both the selectivity and effect of monovalent cation binding in thrombin (*34*). Simple substitution of Pro-225 to Tyr similarly failed to elicit Na^+ activation in tissue-type plasminogen activator (*13*). Addition of the Lys-224 Glu-217 electrostatic pair into SGT aimed to stabilize the Na^+ binding site yet failed to yield a protease responsive to Na^+. The presence of Pro-222 could potentially disrupt the conformation of the 220 loop preventing ion binding, and we then introduced the P222K mutation to yield SGT bearing an identical 220 loop to that observed in FXa. We termed this construct bearing five mutations the 220-loop mutant of SGT (Table 1), and as observed with earlier constructs, it lacked activation by monovalent cations. Introduction of these five mutations into SGT resulted in a 2-fold increase in K_M for Spectrozyme FXa with neglible effect on k_{cat} (Table 3). Clearly, residue 225 and the entire 220 loop of trypsin-like enzymes is not the sole determinant of Na^+ activation.

Crystal Structure of the 220-Loop Mutant of SGT. Hallmarks of Na^+ binding are not evident in the 1.66 Å crystal structure of the 220-loop mutant of SGT (Figure 2). The structure of the 220-loop mutant was solved in conditions similar to those of the wild-type protease: 10 mM calcium acetate, pH 6.1, and 1.8 M ammonium sulfate (*26*, *35*, *36*), in addition to 200 mM NaCl. Crystals grew in the $P6_5$ space group and not the $C222_1$ space group observed with the wild-type or T190P mutant of SGT (*26*). Crystals grown in the presence of significant amounts of Li^+, K^+, Na^+, or Rb^+ as chloride salts yielded identical structural models (data not shown). The carbonyl oxygen atom of Lys-224 is not displaced downward from Asp-189, and the water molecules in the Na^+-binding environment are not suggestive of a bound cation. Similar to the wild-type protease, two water molecules in this region are identified with low *B*-factors. Positions of these two water molecules are shifted relative to the wild-type enzyme presumably due to the altered length of the 186 loop. Notably, the conformation of the 186 loop is not conducive to Na^+ binding in the observed structure. The carbonyl oxygen of Gly-186 in the 220-loop mutant structure is flipped 180° away from the putative Na^+ site similar to that observed in the wild-type enzyme. Valence screening of water molecules in any of the structures obtained did not identify a bound cation (*37*). Further, anomalous scattering was not indicative of a misidentified water molecule for crystals grown in 200 mM KCl or RbCl. No significant differences are observed in the structure when compared to wild-type SGT, in agreement with the minor effects on the enzyme-catalyzed reaction (Table 3). On the basis of the 220-loop crystal structure, we pursued mutagenesis of the 186 loop with the aim of altering the conformation of the carbonyl oxygen atom of residue 186 and generating the proper geometry to support monovalent cation binding.

Mutagenesis of the 186 and 220 Loops Fails To Impart Na^+ Activation. Simultaneous mutagenesis of residues 186 to 188 in the 220-loop mutant of SGT yielded a mutant trypsin whose 186 and 220 loops are identical to FXa (Table

Table 3: Summary of Kinetic Parameters of Mutant SGT Proteases for the Hydrolysis of Spectrozyme FXa[a]

construct	s_0 ($\mu M^{-1} s^{-1}$)	s_1 ($\mu M^{-1} s^{-1}$)	K_d (mM)	fold increase (s_1/s_0)	k_{cat} (s^{-1})	K_M (μM)	s ($\mu M^{-1} s^{-1}$)
wild type					124 ± 4	8.90 ± 0.31	14.0 ± 0.2
220					86.4 ± 1.5	20.5 ± 0.2	4.22 ± 0.04
186 + 220					9.25 ± 0.38	22.2 ± 0.8	0.42 ± 0.01
Y172S + 220					77.7 ± 0.3	285 ± 5	0.27 ± 0.01
Y172S + 186 + 220	0.0047 ± 0.0002	0.018 ± 0.001	300 ± 30	3.8			0.011 ± 0.002
Y172M + 186 + 220	0.0009 ± 0.0001	0.0085 ± 0.0002	200 ± 20	9.4			0.004 ± 0.001
170 + 186 + 220	0.0092 ± 0.0004	0.15 ± 0.01	250 ± 20	16.3			0.070 ± 0.002

[a] Values are given for k_{cat}, K_M, and $s = k_{cat}/K_M$ under standard assay conditions in the presence of 200 mM NaCl. For proteases responsive to NaCl, data were fit to the linkage expression in eq 1 in the text.

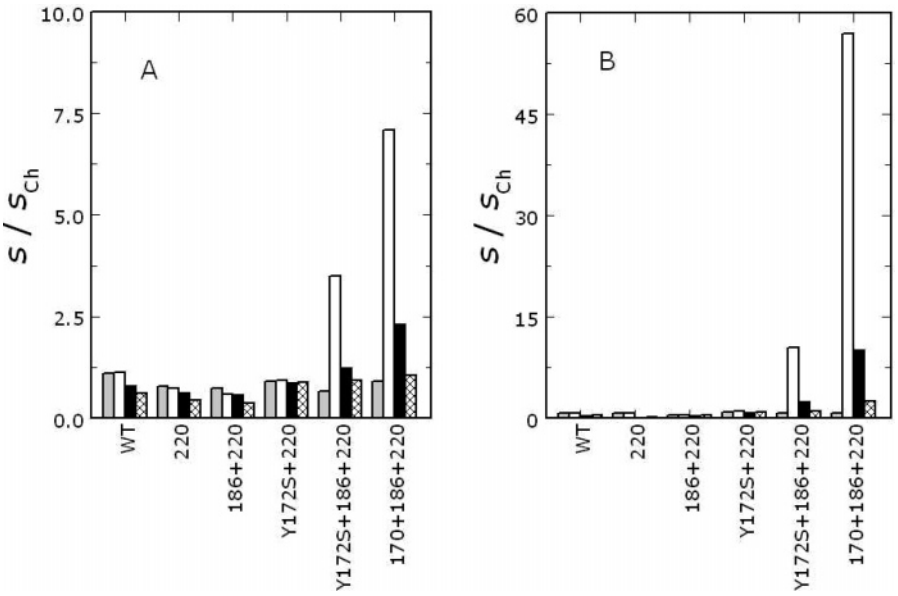

FIGURE 1: Specificity constant $s = k_{cat}/K_M$ of loop mutants of SGT in the presence of LiCl (gray), NaCl (white), KCl (black), or RbCl (hatched), relative to the value in choline (Ch) chloride at (A) 200 mM and (B) 800 mM concentration. The Y172S mutation results in a Na^+-dependent trypsin-like enzyme only when both the 186 and 220 loops of FXa have been introduced. Further mimicry of the 170 loop of FXa leads to significant Na^+ activation. In the presence of 800 mM NaCl, the 170 + 186 + 220 loop mutant of SGT ($s = 0.12\ \mu M^{-1}\ s^{-1}$) is 10-fold less active than the wild-type enzyme ($s = 1.34\ \mu M^{-1}\ s^{-1}$).

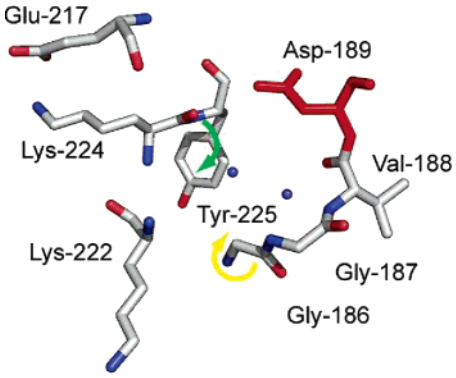

FIGURE 2: Potential Na^+-binding environment of the 220-loop mutant of SGT (PDB ID 2FMJ) reveals the basis for the inability to bind Na^+. Slight changes in the position of two water molecules (blue spheres) are due to the altered length of the 180 loop, but the critical Lys-224 carbonyl does not displace downward from Asp-189 (green arrow). Na^+ binding cannot occur with the 220-loop mutant of SGT given the requirement for 180° rotation of the carbonyl oxygen atom of Gly-186 (yellow arrow). Crystal soaking in up to 400 mM or cocrystallization in 200 mM LiCl, KCl, or RbCl does not alter the observed structural model.

1). Hence, the entire Na^+-binding environment is reproduced in the 186 + 220 loop mutant of SGT. Introduction of these eight changes into the protease failed to produce a Na^+-activated enzyme (Figure 1). Activity (k_{cat}) of the 186 + 220 loop mutant SGT was reduced 9-fold relative to the 220-loop mutant of SGT with almost no effect on the K_M (Table 3). The introduced side chains at positions 186−188 are hydrophilic and should promote the desired conformation of the carbonyl oxygen of residue 186 on the basis of steric hindrance and the inability of these side chains to point inward toward the potential Na^+-binding site. Despite these presumptions, no kinetic effect is evident with this mutant protease. At present, it cannot be determined whether the 180 + 220 loop mutant of SGT does not bind monovalent cations or whether binding occurs but fails to mediate catalytic rate enhancement. The unexpected conclusion drawn from these studies is that engineering the Na^+ activation observed in coagulation proteases into trypsin requires replacements beyond those necessary to reproduce the environment immediately in contact with Na^+.

Mutagenesis of Tyr-172 Results in a Na^+-Activated Protease. We turned to structural information for clues on how Na^+ binding and allosteric transduction is encoded into the protein scaffold. Inspection of the crystal structures of FXa (*38*), aPC (*39*), and thrombin (*22*) reveals a key difference in the residues surrounding the Na^+-binding site relative to other trypsin-like enzymes. Most trypsin-like proteases possess Tyr-172, whose side chain buries within the core of the enzyme (*40*). In turn, the phenolic hydroxyl moiety of Tyr-172 forms a potential H-bond with the amide backbone of Pro-225. Previous mutagenesis studies have demonstrated that residue 172 is an important determinant of primary substrate specificity (*41*). Conversion of Tyr-172 to Trp was crucial for engineering chymotrypsin-like specificity into trypsin. The overlap between residues controlling substrate specificity and Na^+ binding in clotting proteases (*22*, *42*) suggested that residue 172 would also play a key role in Na^+ activation.

Mutagenesis of Tyr-172 of SGT to Ser, the residue found in FXa, in addition to the 186- and 220-loop mutations resulted in a Na^+-activated enzyme (Figure 1). Encouraged by the effect observed upon mutation of Tyr-172 to Ser, we explored the consequences of replacing Tyr-172 in SGT with other residues. These experiments revealed that the nature of residue 172 influences the extent of Na^+ activation. In the presence of both the 186 and 220 loops of FXa, mutagenesis of Tyr-172 to Asp, Asn, His, Met, or Ser resulted in significant Na^+ activation of the engineered protease (Figure 3), with Met showing the largest enhancement of activity (Table 3). Met is present at this position in the Na^+-activated enzyme activated protein C, and its side chain buries in the core of the protease similar to Tyr-172 in trypsin (*39*). The S atom of Met-182 in activated protein

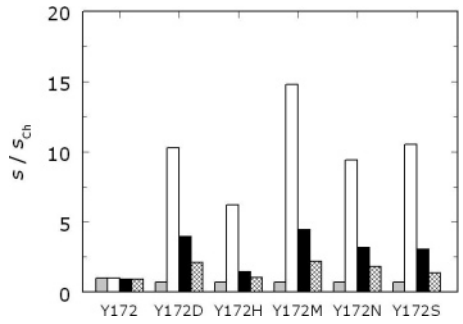

FIGURE 3: Effect of Tyr-172 mutation to Asn, Asp, His, and Met in the 186- and 220-loop mutant of SGT. Shown is the specificity constant $s = k_{cat}/K_M$ in the presence of 800 mM LiCl (gray), NaCl (white), KCl (black), or RbCl (hatched), relative to the value in ChCl. All of the constructed mutants are Na^+-activated enzymes to varying degrees, indicating that absence of the buried Tyr side chain is required for monovalent cation binding and catalytic rate enhancement.

C occupies an identical position to one of the water molecules present in the FXa structure. Notably, the Y172S mutation in combination with the 220-loop mutations does not produce a Na^+-activated protease (Figure 1) and suggests that composition of the 186 loop plays a role in the mechanism. The Y172S + 220 loop mutant has a K_M value 14-fold higher than the 220-loop mutant alone (Table 3). A significant reduction in k_{cat} resulting from mutations of residues 186−188 and the large increase in K_M resulting from the Y172S mutation in SGT explain the poor kinetic properties of the Y172S + 186 + 220 loop mutant of SGT. Our results are strikingly similar to those observed in the conversion of trypsin into a chymotrypsin-like enzyme (*21*, *41*, *43*−*45*). In both instances, mutagenesis of the 186 and 220 loops was initially suspected to introduce the desired catalytic property, yet further work identified a crucial role for residue 172.

Mutagenesis of the 170 Loop Enhances the Effect of Na^+. Further mimicry of the entire 170 loop of FXa resulted in an enzyme that is potently activated by Na^+, with the value of $s = k_{cat}/K_M$ increasing almost 60-fold relative to the inert cation choline (Figure 1). The final construct bears 14 amino acid substitutions (Figure 4A). In trypsin, Tyr-172 buries within the core of the enzyme pulling the 170 loop closer to the enzyme and effectively eliminating the S4 pocket (Figure 4B) (*26*). In contrast, absence of Tyr-172 facilitates an open conformation of the 170 loop and defines the S4 pocket of the enzyme (Figure 4C) (*3*, *38*, *46*). The E180M mutation was also included in the 170 + 186 + 220 loop mutant of SGT to ensure proper H-bonding and positioning of the 170 loop (Table 1). Three water molecules occupy the position equivalent to the buried Tyr side chain. The recently solved structure of human FXa at 1.64 Å clearly identifies the positions of these three buried water molecules (*47*). A network of eight H-bonds mediated through water molecules links the amide and carbonyl groups of Tyr-225 to the

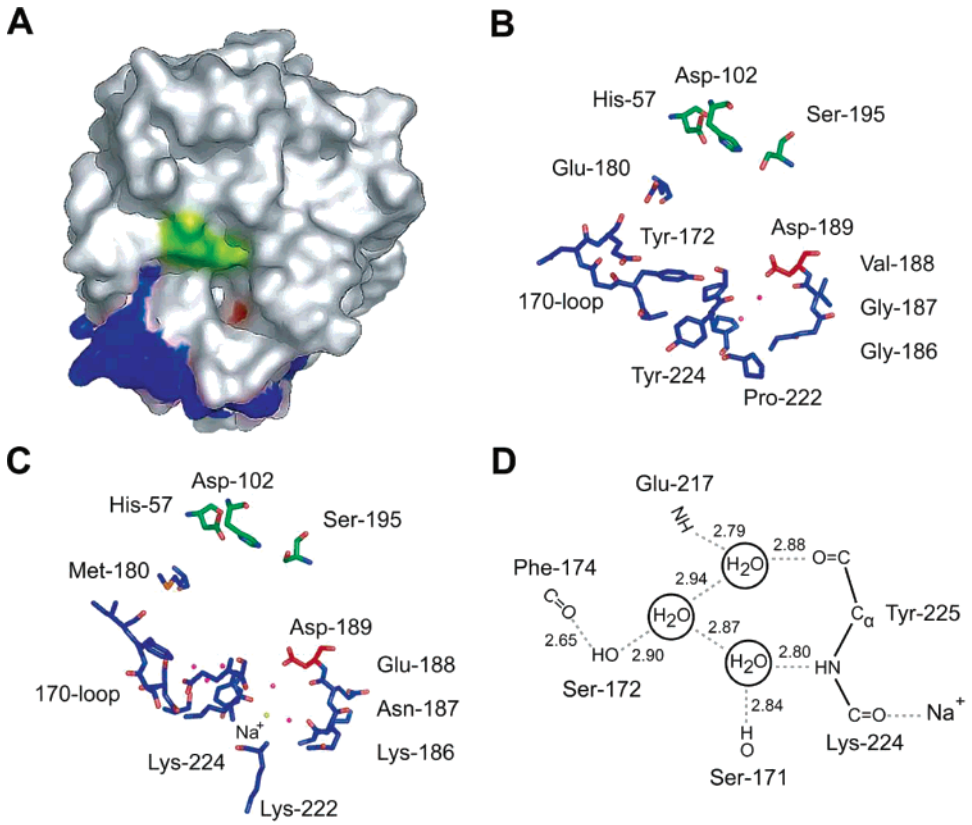

FIGURE 4: (A) Surface model of the residues modified in SGT (blue) to reproduce Na^+ activation similar to FXa. Mutations introduced form a network of contacts that run underneath the enzyme active site and link the Na^+ site to the 170 loop (S4 pocket). Residues altered do not contact Asp-189 (red) at the base of the S1 pocket or the catalytic triad (His-57, Asp-102, and Ser-195) (green). (B) Tyr-172 in SGT (PDB ID 1OS8) is buried in the core of the enzyme similar to that observed in many trypsin-like enzymes. (C) In FXa (PDB ID 2BOK), the Na^+ ion (yellow) is coordinated by four carbonyl oxygen atoms donated by the 186 and 220 loops and is directly linked to the critical Asp-189 side chain through one of two water molecules in the coordination shell (magenta spheres). Absence of Tyr-172 allows the 170 loop to adopt a conformation that defines the S4 pocket of the enzyme, and the vacated space is occupied by three water molecules. (D) Schematic diagram of the H-bonding network in FXa (PDB ID 2BOK) that links the Na^+-binding site to the 170 loop via three water molecules.

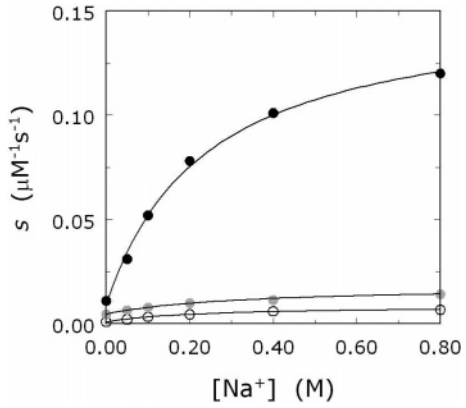

FIGURE 5: Specificity constant $s = k_{cat}/K_M$ as a function of $[Na^+]$ for the Y172M + 186 + 220 (open circle), Y172S + 186 + 220 (shaded circle), and 170 + 186 + 220 (solid circle) mutants of SGT. Na^+-activated mutants of SGT possess apparent K_{d,Na^+} significantly higher than reported for FXa or thrombin. Continuous lines were drawn according to the linkage expression (eq 1 in the text) and the best-fit parameter values given in Table 3.

hydroxyl groups of Ser-171 and Ser-172, the amide backbone of Glu-217, and the carbonyl oxygen of Phe-174 in FXa (H-bond lengths of 2.83 ± 0.09 Å) (Figure 4D). We suggest that mutations introduced in the 170 loop in our construct play a similar role to that observed in FXa and stabilize the buried water molecule network resulting in a more potent effect on catalytic rate enhancement in the presence of Na^+. Titration of the constructed Na^+-activated proteases demonstrated weak cation binding characteristics (Figure 5), with values of K_d for Na^+ binding (Table 3) significantly higher than those reported for thrombin ($K_{d,Na^+} = 14$ mM) (*22*) or FXa ($K_{d,Na^+} = 39$ or 9.5 mM in the presence of Ca^{2+}) (*18*). Nonetheless, the activating effect of Na^+ is pronounced, proving that essential determinants for Na^+ binding and allosteric transduction have been introduced for the first time in a serine protease.

Loss of catalytic activity of the final construct relative to wild-type enzyme, despite its acquired Na^+ activation, is not surprising given the extensive mutagenesis (alteration of 13 residues in combination with a 1-residue deletion) applied to SGT. The final construct is 200-fold less active than wild type (Table 3), although the presence of Na^+ activation reduces the difference to only 10-fold ($s = 0.12$ μM^{-1} s^{-1} mutant vs $s = 1.34$ μM^{-1} s^{-1} wild type) at 800 mM NaCl (Figure 1). Improvement of catalytic activity in SGT is an unrealistic expectation as the value of $s = k_{cat}/K_M$ is close to the diffusion-controlled rate for proteins in aqueous solutions (*48*). The importance of engineering Na^+ activation in SGT should be appreciated as a proof of principle and placed in the context of the large number of proteases that have intrinsically poor activity toward substrate, such as tPA or several complement enzymes. Future engineering studies will reveal if the strategy used for SGT has wider applicability and may indeed increase the catalytic activity of poor enzymes. In the absence of enhanced catalytic activity, Na^+ activation provides a mechanism for allosteric regulation which many proteases including SGT lack. Such regulation may bring about changes in specificity and drastic shifts in biological activities as demonstrated by thrombin (*49*).

Role of Pro-225 and Tyr-172 in Trypsin-like Proteases. Previous studies on protease specificity have drawn attention to the highly cooperative nature of the recognition process, with several domains of the enzyme being involved in substrate binding beyond the residues in immediate contact with substrate (*4*, *41*, *46*). These studies have also demonstrated that the engineering strategy to convert specificity is not general and important differences exist in different systems (*45*). In agreement with these findings, engineering Na^+ binding and activation into trypsin have proved to be a task of comparable difficulty and complexity. Circumstantial evidence in the developmental protease easter suggests that simple replacement of Pro-225 with Tyr is sufficient to produce a more active enzyme (*50*), as though Na^+ activation could be introduced in easter by a single amino acid substitution (*51*). This is obviously not the case in tissue-type plasminogen activator (*13*) and SGT, where mutation of Pro-225 fails to elicit Na^+ binding and activation. Introduction of all residues contacting Na^+ in SGT does not ensure Na^+ activation. Additional mutagenesis of residue 172 and its environment appears to be necessary for a significant allosteric enhancement of catalytic activity to be introduced in trypsin. Whether such strategy has general validity remains to be demonstrated by future mutagenesis studies in other systems.

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