Ser\textsuperscript{214} Is Crucial for Substrate Binding to Serine Proteases*

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Highly conserved amino acids that form crucial structural elements of the catalytic apparatus can be used to account for the evolutionary history of serine proteases and the cascades into which they are organized. One such evolutionary marker in chymotrypsin-like proteases is Ser\textsuperscript{214}, located adjacent to the active site and forming part of the primary specificity pocket. Here we report the mutation of Ser\textsuperscript{214} in thrombin to Ala, Thr, Cys, Asp, Glu, and Lys. None of the mutants seriously compromises the active site catalytic function as measured by the kinetic parameter $k_{\text{cat}}/K_m$. However, the least conservative mutations result in large increases in $K_m$, because of lower rates of substrate diffusion into the active site. Therefore, the role of Ser\textsuperscript{214} is to promote the productive formation of the enzyme-substrate complex. The S214C mutant is catalytically inactive, which suggests that during evolution the TCN→AGY codon transitions for Ser\textsuperscript{214} occurred through Thr intermediates.

Serine proteases have been classified into evolutionarily and structurally unrelated clans (1), which nevertheless maintain a strictly conserved active site geometry among their catalytic Ser, His, and Asp residues. This shared catalytic structure suggests that common architectural motifs can be found in the molecular design of active sites utilizing a Ser-His-Asp triad. The identification of discrete evolutionary markers encountered in the sequences contributing to the catalytic apparatus has shed light on both crucial structural motifs and evolutionary events in the history of serine protease families (2). The identification of a single nucleotide evolutionary marker at the respective protease clan (2). Each of these residues (Ser\textsuperscript{214}, Ser\textsuperscript{125}, and Cys\textsuperscript{341}) serves as an evolutionary marker and appears to contribute to catalytic function in its respective protease clan (2).

The role of Ser\textsuperscript{214} in chymotrypsin-like proteases has been addressed in previous studies (5). The trypsin mutants S214E and S214K disrupted the environment of the catalytic Asp\textsuperscript{102} as demonstrated kinetically and crystallographically. However, the S214A mutant described in the same report had improved specificity ($k_{\text{cat}}/K_m$) compared with wild type, calling into question the requirement for the Ser\textsuperscript{214} side chain in catalysis. More importantly, previous studies on trypsin have not addressed how mutations of Ser\textsuperscript{214} affect the individual steps of the catalytic mechanism of substrate hydrolysis and have left the precise origin of the perturbation unanswered.

Here we report the effect of several substitutions (Ala, Asp, Glu, Lys, Thr, and Cys) of Ser\textsuperscript{214} in thrombin by dissecting the kinetics of substrate hydrolysis using a novel and powerful approach (10). Considering the TCN→AGY Ser codon transitions that must have taken place at Ser\textsuperscript{214} (2), it has now become relevant to also generate S214T and S214C mutants as the codons for Thr and Cys (ACN and TGY, respectively) represent intermediates in the single nucleotide transitions between TCN and AGY. In addition to trypsin, thrombin is a most relevant system for studying the contribution of Ser\textsuperscript{214} to serine protease catalysis. Thrombin is a Na\textsuperscript{+}-activated enzyme, which adds complexity to the mechanism of substrate recognition, and has a more stringent specificity profile than trypsin that may be more sensitive to mutations that alter $k_{\text{cat}}$ or $K_m$. Hence, the mutation of Ser\textsuperscript{214} in thrombin would reveal whether that evolutionary marker is linked to Na\textsuperscript{+} binding in serine proteases and would test whether previous results on trypsin can be generalized to the majority of chymotrypsin-like proteases.

MATERIALS AND METHODS

Site-directed mutagenesis of human α-thrombin was carried out in a HPC4-pNUT expression vector using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Expression of thrombin mutants was carried out in baby hamster kidney cells as described previously (11). Mutants were activated with the prothrombinase complex between 40 and 60 min at 37 °C. Enzymes used in the activation were supplied by Enzyme Research (South Bend, IN). Mutants were purified to homogeneity by fast protein liquid chromatography using Resource Q and S columns with a linear gradient from 0.05 to 0.5 M choline chloride (ChCl), 5 mM MES, pH 6.0, at room temperature. Active site concentrations were determined by titration with hirudin.

The substrate H-D-Phe-Pro-Arg-p-nitroanilide (FPR) was synthesized by Midwest Biotech (Carmel, IN). Individual rate constants defining the mechanism of substrate hydrolysis by serine proteases were extracted from the values of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ obtained as a function of temperature (10) from 5 to 45 °C under solution conditions of 5 mM Tris, 0.1% PEG-8000, 200 mM NaCl, pH 8.0. The values for the Na\textsuperscript{+}-free slow form of wild type were obtained by replacing NaCl with ChCl in the
buffer. Substrate hydrolysis entails the binding of substrate S to the enzyme E with a second-order rate constant \( k_s \). After the formation of the enzyme-substrate complex ES, the complex can either dissociate back into the solution with a rate constant of \( k_1 \) or become acylated with a rate constant of \( k_2 \). The portion of substrate distal to the scissile bond, \( P^* \), is released at this stage. The acyl intermediate EP is subsequently hydrolyzed to release the portion of substrate proximal to the scissile bond, \( P' \), with a rate constant of \( k_3 \) as shown in Scheme 1.

\[
s = -\frac{k_1}{k_2} \exp \left( \frac{E_1 + E_0}{R} \left( \frac{1}{T} - \frac{1}{T_0} \right) \right) + \frac{k_2}{k_3} \exp \left( -\frac{E_0}{R} \left( \frac{1}{T} - \frac{1}{T_0} \right) \right)
\]

(Eq. 1)

\[
h_{cat} = \frac{k_1}{k_2} \exp \left( \frac{E_1 + E_0}{R} \left( \frac{1}{T} - \frac{1}{T_0} \right) \right)
\]

(Eq. 2)

where \( E_0 \) is the activation energy associated with the rate constant \( k_1 \) or \( k_2 \), \( R \) is the gas constant, \( T \) is the absolute temperature, and the values refer to \( T_0 = 298.15 \) K. The measurements of \( s \) and \( h_{cat} \) as a function of temperature can resolve all the parameters in Equations 1 and 2 provided the plots show curvature. In the event of \( k_1 \)>>\( k_2 \) (\( R/T_1 \)) as typically observed for thrombins, \( h_{cat} \) cannot be resolved and the plot of \( logh_{cat} \) versus \( 1/T \) is a straight line. When \( k_1 \) cannot be determined because of high \( K_m \) values, a plot of \( logS \) versus \( 1/T \) yields \( k_1 \), the ratio \( a = k_1/k_{cat} \), and the difference \( E_a = E_1 - E_3 \). Again provided the plots show curvature.

Physiologic substrates and inhibitors were studied under the conditions of 5 mM Tris, 0.1% PEG-8000, 200 mM NaCl, pH 8.0 at 25 °C. The cleavage of fibrinogen leading to the release of fibrinopeptides A and B and cleavage of the protease-activated receptor (PAR) peptides PAR1, PAR2, and PAR4 were carried out by HPLC as described previously (12). The activation of protein C in the presence of 5 mM CuCl_2 and 10 nm rabbit thrombomodulin was measured and analyzed as reported elsewhere (11). Antithrombin inhibition was studied by changing the concentration of unfractionated heparin to determine the optimal inhibition of each mutant (13), monitored by following progress curves of FPR hydrolysis in which the antithrombin concentration was varied. The observed rate of inhibition, \( k_{cat} \), was calculated as described previously (11).

Equilibrium dissociation constants for Na\(^+\) binding were determined by fluorescence titration using a FluoroMax-3 SPEX spectrophotometer. Fluorescence titrations took place under experimental conditions of 5 mM Tris, 0.1% PEG-8000, 800 mM ionic strength, pH 8.0 at 10 °C. The temperature was chosen to maximize both the intrinsic fluorescence of the enzyme and the fluorescence increase induced by Na\(^+\) binding. Titrations were carried out by adding aliquots of thrombin in 800 mM NaCl to a solution containing the enzyme in 800 mM ChCl. Ionic strength (800 mmo) and enzyme concentration (200 nm) were held constant, whereas [Na\(^+\)] was varied. Excitation was at 295 nm, and emission was measured at 333 nm. The value of thrombin intrinsic fluorescence, \( F_0 \), as a function of [Na\(^+\)] was fit according to the Equation 3,

\[
F = \frac{F_0 + F_1 [Na^+]_{cat}}{1 + [Na^+]_{cat} K_{cat}}
\]

(Eq. 3)

where \( F_0 \) and \( F_1 \) are the values of \( F \) in the absence and under saturating [Na\(^+\)] and \( K_{cat} \) is the equilibrium dissociation constant for Na\(^+\) binding.

Na\(^+\) dissociation constants were alternately determined by following the linkage between the \( k_{cat}/K_m \) for FPR hydrolysis and [Na\(^+\)] under conditions of 50 mM Tris, 0.1% PEG-8000, pH 8.0 at 25 °C. The value of \( s = k_{cat}/K_m \) was fit according to the Equation 4 (11),

\[
s = \frac{s_0 + s_1 [Na^+]_{cat}}{1 + [Na^+]_{cat} K_{cat}}
\]

(Eq. 4)

where \( s_0 \) and \( s_1 \) are the values of \( k_{cat}/K_m \) in the absence and under saturating [Na\(^+\)], pertaining, respectively, to the Na\(^+\)-free slow and Na\(^+\)-bound fast forms and \( K_{cat} \) is the equilibrium dissociation constant for Na\(^+\) binding.

Temperature melting curves were measured under conditions of 200 mM NaCl or ChCl, 5 mM Tris, pH 8.0, 0.1% PEG-8000 over the temperature range of 15–80 °C. Denaturation was monitored by following absorbance at 280 nm. Experiments were conducted using sufficient enzyme to generate an absorbance of ~0.05 absorbance units at 15 °C. Temperature was then gradually increased to 80 °C at a rate of 1.5 °C/min. The melting temperature was calculated from the midpoint of the melting transition.

**RESULTS**

Ser \(^{214}\) thrombin mutants have reduced catalytic activity as measured by FPR hydrolysis with the most significant reductions corresponding to the mutants carrying the largest side chains at position 214 (Table 1). Although the Ala, Thr, and Lys mutants have reduced \( k_{cat} \) compared with wild type, the Asp
and Glu mutants have increased \( k_{\text{cat}} \). Therefore, changes in \( k_{\text{cat}} \) do not correlate with the size of the side chain placed at position 214. However, the presence of a residue other than Ser at position 214 dramatically increases \( K_m \). The least conservative mutant, S214K, shows a 1000-fold increase in \( K_m \) with only a 3-fold drop in \( k_{\text{cat}} \). Only limited kinetic data could be collected for the S214C mutant (see below).

The impact of Ser\(^{214} \) on \( K_m \) rather than \( k_{\text{cat}} \) suggests a major role in substrate binding, which is confirmed by the individual rate constants gleaned from temperature dependence studies of \( k_{\text{cat}}/K_m \) and \( k_{\text{cat}} \) for FPR hydrolysis (Fig. 1 and Table I). The increases in \( K_m \) are primarily attributable to decreases in the rate of association \( k_1 \), which are most significant for the conservative mutations. The values of \( k_1 \) for the Ala and Thr mutants approach that of the slow form of wild type, whereas those of the Asp, Glu, and Lys mutants deviate more to the downside and underline more drastic structural perturbations of the accessibility of the active site. The reductions in \( k_1 \) tend to be linked to significant decreases in the activation energy, \( E_1 \), for this step. This suggests that wild-type thrombin undergoes an induced fit rearrangement on substrate binding that cannot be duplicated fully in the Ser\(^{214} \) mutants. The integrity of the Ser side chain ensures this structural change and optimizes the productive collision of substrate with the active site. When the catalytic rate constants are considered, all mutants are like wild type in that \( k_1 \gg k_2 \) and the log\( k_{\text{cat}} \) versus \( 1/T \) plot is linear. Interestingly, none of the mutants compromises \( k_{\text{cat}} \) significantly, and the presence of a negatively charged side chain at position 214 actually increases the acylation rate and \( k_{\text{cat}} \) relative to wild type.

There is an apparent dichotomy in the functional behavior of the mutants of Ser\(^{214} \), because they tend to behave like the Na\(^+\)-free slow form of wild type when the rate of substrate diffusion into the active site is considered but mimic more closely the Na\(^+\)-bound fast form in their catalytic rate constants. This finding suggests that Ser\(^{214} \) is energetically linked to Na\(^+\) binding especially in the steps that control substrate binding and formation of the enzyme-substrate complex. This expectation is confirmed by the inspection of the Na\(^+\) binding affinities of the Ser\(^{214} \) mutants as measured directly by intrinsic fluorescence titration (Fig. 2) that are compromised to a significant extent (up to 50-fold). Consistent with these findings, Na\(^+\) binding partially rescues the Ser\(^{214} \) mutants (Table II) because the value of the specificity constant \( s_0 \) in the absence of Na\(^+\) (slow form) is more compromised relative to wild type than the value \( s_1 \) obtained under saturating [Na\(^+\)] (fast form). This finding demonstrates that the deleterious effect of replacing Ser\(^{214} \) is felt more on the Na\(^+\)-free slow form.

Ser\(^{214} \) thrombin mutants also demonstrate impaired cleavage of physiologic substrates (Table III). Specificity constants for the release of fibrinopeptides A and B, which measure the ability of thrombin to cleave fibrinogen to form a fibrin clot, are compromised to a similar degree. Protein C activation, an anticoagulant activity, is slightly less compromised with the exception of S214K. The cleavage of PAR peptides is reduced to a similar extent for PAR1, PAR3, and PAR4. Because of the severely impacted catalytic efficiency of the S214K mutant, specificity constants toward PAR3 and PAR4 could not be measured. The least conservative mutants show the greatest reduction in antithrombin inhibition as measured by the rate of inactivation \( k_{\text{inact}} \). The mutants require slightly higher concent-
trations of heparin relative to wild type to ensure inhibition. The optimal heparin concentration for wild type is 0.5 USP units/ml, whereas the majority of the mutants require 1–2 USP units/ml heparin. Selected S2 and S3 specificity subsite mutants of thrombin also demonstrate increased requirements for heparin (13).

The S214C mutant could not be characterized extensively. During the first attempt at activation of S214C prethrombin-1, the mutant thrombin appeared to aggregate in solution. Despite this result, it was possible to purify a fraction of the mutant whose absorbance indicated a concentration of 700 nM. In spite this result, it was possible to purify a fraction of the mutant. However, it was not possible to purify a fraction of the mutant whose absorbance indicated a concentration of 700 nM.

During the first attempt at activation of S214C prethrombin-1, heparin (13). The instability of S214C does not result from impaired Na+/H11001 transport but rather from the presence of the extra Cys at position 214, which causes improper intramolecular disulfide bonds to form, resulting in an incorrectly folded enzyme. It is possible that the presence of the extra Cys at position 214 causes improper intramolecular disulfide bonding, and that the unpaired Cys in the enzyme, intermolecular disulfide bonding appears improbable. As the unpaired Cys in all of the activated material was catalytically inactive. The loss of activity seen for S214C is puzzling. Polyacrylamide gel electrophoresis of aliquots from the activation reactions and purified fractions does not indicate proteolysis. As the unpaired Cys of the enzyme, intermolecular disulfide bonding appears improbable. It is possible that the presence of the extra Cys at position 214 causes improper intramolecular disulfide bonds to form, resulting in an incorrectly folded enzyme.

Temperature denaturation experiments were used to assess the relative stabilities of wild type, S214T, and S214C. The melting temperatures for the S214C and S214T mutants are 10 and 5 °C, respectively, lower than that for wild type (62 °C). The instability of S214C does not result from impaired Na+ binding as wild type has the same melting temperature in both NaCl (fast form) and ChCl (slow form) solutions. Ser214 appears to be an important residue for the folding of thrombin. The mutation to Thr slightly destabilizes the fold, but producing a free disulfide at this position destabilizes the fold enough to produce an inactive and presumably misfolded enzyme.

**DISCUSSION**

The results presented here on the role of Ser214 in serine proteases extend those reported previously in the case of trypsin (5) that have left several basic questions unanswered. In particular, previous studies have characterized the kinetic origin of the perturbations only in terms of \( k_{cat} \) and \( K_m \). Our study has examined the effect of Ser214 mutations on each step of the catalytic mechanism of substrate hydrolysis and has identified the precise events in which Ser214 is involved, namely the optimization of substrate diffusion in the active site, the induced fit rearrangement of the enzyme-substrate complex, and the unanticipated energetic linkage with Na+ binding.

Ser214 is the only residue among the four with side chains in the active site that is not required for proper function of the Ser-His-Asp charge relay system. As such, it presents an opportunity to probe the environment of the active site around Asp102. The active site is extremely tolerant to substitution at position 214 as the presence of a buried Lys produces only a 3-fold reduction in \( k_{cat} \). However, it should be noted that in trypsin, the reduction in \( k_{cat} \) was more dramatic. In α-lytic protease, the mutation of Ser214 to Ala brought about a 4900-fold reduction in specificity, but Epstein and Abeles (14) surmise that the catalytic function remained intact.

Ser214 seems to play a minimal role in governing the catalytic rate reflected by the minor effects on \( k_{cat} \). Crystal structures of trypsin (5) and duodenase (6) have led to the hypothesis that Ser214 helps position Asp102 of the charge relay system for optimal catalysis. Slight reductions in \( k_{cat} \) for S214A, S214T, and S214K lend support to this idea but prove that Ser214 is not essential for effective catalysis. Catalytically, the most interesting mutants are S214D and S214E, which display approximately 3-fold reduction in \( k_{cat} \). However, it should be noted that in trypsin, the reduction in \( k_{cat} \) was more dramatic. In α-lytic protease, the mutation of Ser214 to Ala brought about a 4900-fold reduction in specificity, but Epstein and Abeles (14) surmise that the catalytic function remained intact.

Ser214 is crucial for substrate binding in serine proteases.

The parameters \( s_0 \) and \( s_1 \) are the values of \( k_{cat}/K_m \) for the slow and fast forms, respectively, whereas \( K_m \) is the dissociation constant for Na+ binding (see also Equation 4 under "Materials and Methods").

### Table II

|                | WT | S214A | S214D | S214E | S214K | S214T |
|----------------|----|-------|-------|-------|-------|-------|
| \( s_0 \) (μM⁻¹ s⁻¹) | 4.8 ± 0.1 | 0.093 ± 0.004 | 0.014 ± 0.001 | 0.011 ± 0.001 | 0.0016 ± 0.0001 | 0.065 ± 0.003 |
| \( s_1 \) (μM⁻¹ s⁻¹) | 90 ± 3 | 16 ± 1 | 12 ± 1 | 44 ± 5 | 300 ± 30 | 1800 ± 200 |
| \( K_m \) (mM)     | 44 ± 5 | 300 ± 30 | 1800 ± 200 | 700 ± 100 | 230 ± 20 | 810 ± 30 |

### Table III

|                | Wild type | S214A | S214D | S214E | S214K | S214T |
|----------------|-----------|-------|-------|-------|-------|-------|
| FpA (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 11 ± 1 | 2.0 ± 0.1 | 0.52 ± 0.04 | 0.079 ± 0.003 | 0.0047 ± 0.0001 | 1.3 ± 0.1 |
| FpB (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 6.2 ± 0.3 | 0.90 ± 0.09 | 0.26 ± 0.05 | 0.042 ± 0.008 | 0.0022 ± 0.0002 | 0.67 ± 0.08 |
| Protein C (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 42 ± 3 | 17 ± 1 | 3.4 ± 0.3 | 0.71 ± 0.06 | 0.0061 ± 0.0005 | 27 ± 1 |
| PAR1 (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 14 ± 1 | 2.6 ± 0.1 | 0.68 ± 0.09 | 0.15 ± 0.02 | 0.012 ± 0.001 | 1.8 ± 0.1 |
| PAR3 (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 0.46 ± 0.02 | 0.067 ± 0.004 | 0.044 ± 0.003 | 0.0031 ± 0.0001 | n.d. a | 0.063 ± 0.003 |
| PAR4 (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 0.40 ± 0.02 | 0.042 ± 0.002 | 0.016 ± 0.003 | 0.0011 ± 0.0003 | n.d. a | 0.028 ± 0.002 |
| Antithrombin III (\( k_{cat}/K_m \) in μM⁻¹ s⁻¹) | 4.7 ± 0.1 | 0.46 ± 0.01 | 0.40 ± 0.01 | 0.016 ± 0.003 | 0.0062 ± 0.0006 | 0.45 ± 0.01 |

a Too small to detect.
Ser²¹⁴ Is Crucial for Substrate Binding in Serine Proteases

dinulating the Na⁺/H₂O molecules of the S₁ channel (19), and perturbations of Ser 214 by Na⁺/H₂O proteases, the residue analogous to Ser 214 is frequently the located near the Na⁺ binding site. For example, the mutations of Trp⁶⁰⁴ and Trp²¹⁵ located 17 and 10 Å away from the Na⁺ site practically abolish Na⁺ binding (17, 18). The backbone atoms of Trp²¹⁵ adjacent to Ser²¹⁴ make contact with two water molecules of the S₁ channel (19), and perturbations of Ser²¹⁴ may be transmitted via Trp²¹⁵ to the four water ligands coordinating the Na⁺ ion (15, 16). The significantly reduced Na⁺ binding of Ser²¹⁴ mutants indicates the long range influence of that residue on the S₁ pocket structure. Also, the structural perturbation caused by the replacement of Ser²¹⁴ affects predominantly the Na⁺-free slow form (Table II) and is corrected by Na⁺ binding by virtue of the reciprocity of the linkage between this residue and Na⁺ as seen for mutants of residues Trp⁶⁰⁴ and Trp²¹⁵ (17, 18).

The S₂¹⁴C mutant is 95% inactive. The residue presumably engages in an improper disulfide bond with another Cys residue; however, there is no outstanding candidate for this other residue because the nearest Cys to Ser²¹⁴ is 9 Å away. Temperature denaturation studies also suggest that Ser²¹⁴ plays a role in thrombin stability. Among αβ-hydrolase-fold serine proteases, the residue analogous to Ser²¹⁴ is frequently the unpaired Cys³⁴¹. A subtilisin-like protease with Cys at the analogous position 125 also exists (2). Therefore, unpaired Cys residues are easily tolerated adjacent to serine protease active sites except in the chymotrypsin-like clan. There are several instances of Thr usage at position 214 including human complement factor D. In factor D, Thr and Ser are interchangeable (20), indicating the viability of Thr at position 214. The unsuitability of Cys and the feasibility of Thr at position 214 of thrombin suggest the codon switch for Ser²¹⁴ from TCN to AGY if occurring by single-nucleotide transitions utilized a Thr intermediate.

The case for a true catalytic tetrad, at least in clan PA, now seems weaker than originally suggested by sequence data (2). Among chymotrypsin-like proteases, Ser²¹⁴ appears to have been conserved because of its role in substrate recognition, ensuring optimal diffusion into the active site and proper induced fit rearrangement of the enzyme-substrate complex. Upon first glance, this function may not seem as crucial as that performed by the catalytic nucleophile Ser¹⁹⁵, but Ser²¹⁴ is conserved almost as strongly as Ser¹⁹⁵, highlighting the evolutionary and physiologic importance of tight substrate binding (21) in the vast majority of serine proteases.

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