Energetic and Structural Consequences of Perturbing Gly-193 in the Oxyanion Hole of Serine Proteases*

Received for publication, March 30, 2005, and in revised form, May 6, 2005* Published, JBC Papers in Press, May 12, 2005, DOI 10.1074/jbc.M503499200

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The oxyanion hole of serine proteases is formed by the backbone N atoms of the catalytic Ser-195 and Gly-193 and engages the backbone O atom of the P1 residue of substrate in an important H-bonding interaction. The energetic contribution of this interaction in the ground and transition states is presently unknown. Measurements of the individual rate constants defining the catalytic mechanism of substrate hydrolysis for wild-type thrombin and trypsin and their G193A and G193P mutants reveal that Gly-193 is required for optimal substrate binding and acylation. Crystal structures of the G193A and G193P mutants of thrombin bound to the active site inhibitor H-D-Phe-Pro-Arg-CH2Cl document the extent of perturbation induced by the replacement of Gly-193. The Ala mutant weakens the H-bonding interaction of the N atom of residue 193, whereas the Pro substitution abrogates it altogether with additional small shifts of the protein backbone. From the kinetic and structural data, we estimate that the H-bonding interaction in the oxyanion hole contributes a stabilization of the ground and transition states of >1.5 kcal/mol but <3.0 kcal/mol. These results shed light on a basic aspect of the enzyme-substrate interaction in the entire family of trypsin-like serine proteases.

Serine proteases catalyze the hydrolysis of peptide bonds using the triad His-57/Asp-102/Ser-195 (chymotrypsinogen numbering) (1, 2). Binding of substrate to the active site is stabilized by a network of H-bonds, five of which are highly conserved and ensure the efficient hydrolysis of amide bonds. Of these H-bonds, two stabilize an anti-parallel β-sheet between Gly-216 and the P3 residue of substrate, whereas the other three involve the backbone N and O atoms of the P1 residue of substrate. The N atom engages the backbone O atom of Ser-214, often referred to as the “fourth” member of the catalytic machinery (3). The O atom of the P1 residue, on the other hand, makes two H-bonding interactions with the backbone N atoms of the catalytic Ser-195 and Gly-193 in the so-called “oxyanion hole.” The role of this region of the enzyme is to stabilize the developing negative charge on the O atom of substrate during formation of the tetrahedral intermediate (4). The lack of a side chain at position 193 allows correct positioning of the amido hydrogen to form the requisite H-bond with the oxyanion substrate. Indeed, Gly is highly conserved at position 193 in serine proteases, but few exceptions do exist and are associated with perturbed substrate hydrolysis and resistance to inhibition (5–8).

Previous studies have addressed the important question of the energetic involvement of the oxyanion hole in substrate recognition. In subtilisin, one of the H-bonding group in the oxyanion hole is provided by the side chain of Asn-155 (9). Mutation of Asn-155 to the isosteric Leu, devoid of H-bonding capabilities, produces a derivative with reduced $k_{cat}/K_m$ due to a significant (200-fold) decrease of $k_{cat}$ (9, 10). The absence of effect on $K_m$ has been interpreted as supporting the role of the H-bonding network of the oxyanion hole in stabilization of the transition state. In Escherichia coli type I signal peptidase, one of the H-bonding group in the oxyanion hole is the hydroxyl of Ser-88 (11). Mutation of Ser-88 to Ala drastically impairs the $k_{cat}/K_m$ due to a significant (>2000-fold) decrease of $k_{cat}$. As for subtilisin, the effect on $K_m$ is minimal and suggests that the H-bonding contribution of the oxyanion hole stabilizes predominately, if not exclusively, the transition state. In the case of serine proteases such as trypsin and chymotrypsin, the two H-bonding donors of the oxyanion hole are backbone N atoms, and it is not possible to perturb them directly as in the case of subtilisin and E. coli type I signal peptidase. Any substitution at Ser-195 would result in an inactive enzyme, and mutations of Gly-193 may affect other properties of the active site beyond the H-bonding interaction provided by this residue backbone. Hence, for trypsin-like enzymes, no conclusive evidence currently exists about the role of the oxyanion hole in stabilizing exclusively the transition state. In the case of clotting factor IXa, the mutant G193E shows perturbation of both $K_m$ and $k_{cat}$ (8), suggesting a role for Gly-193 in stabilization of both the ground and transition states. However, no energetic estimate of the role of Gly-193 in these interactions has been reported thus far. In the case of subtilisin, the contribution to transition state stabilization was estimated to be 3.7 kcal/mol (10). However, the absence of effect on ground state stabilization was inferred from the values of $K_m$, which may not be an accurate measure of the equilibrium binding constant $K_d$ (12). Hence, even for subtilisin, questions persist on the role of the oxyanion hole in stabilization of the ground state.

Given the central importance of the transition state stabilization hypothesis in the mechanism of substrate hydrolysis by serine proteases (2) and the preeminent role played by trypsin- and chymotrypsin-like enzymes in biological processes in health and disease (13), we have decided to explore the role of Gly-193 in thrombin and trypsin using detailed kinetic and structural analysis. Perturbation of the H-bonding contribution from Gly-193 was achieved with two mutations, G193A and...
G193P. The Ala mutant was made as reference to establish the effect of introducing the smallest chiral center on the H-bonding capability of residue 193. The Pro mutant was made to completely abrogate the H-bonding capability of residue 193. In each case, we have resolved the crystal structures of the G193A and G193P mutants to directly gauge the extent of structural perturbation produced by the mutations on the oxyanion hole. The combination of kinetic, thermodynamic, and structural data offers a quantitative assessment of the role of Gly-193 and the oxyanion hole in substrate recognition.

MATERIALS AND METHODS

Construction of the Trypsinogen Vector—The pPICZα vector containing anionic rat trypsinogen was constructed using the riboloning technique (14). The trypsinogen gene was amplified from a plasmid present in the laboratory by standard PCR using the following primers: 5′-TTTCCCGTGATGATGACAGATCTTGAGG-3′ and 5′-CTATGGCCAGCAATTGTGTCCTGAATCCAGTC-3′. The gene was prepared for insertion into the pPICZα vector immediately downstream of the factor signal sequence using two riboprimers and two "band-aid" primers. KlenTaq long and accurate DNA polymerase (14) was used at pH 9.2 for the PCR. The pPICZα "target" was prepared using two riboprimers, which amplified with an insertion gap for the trypsinogen gene. RNase A was digested by addition of proteinase K (Sigma). The prepared pPICZ vector and the prepared trypsinogen gene insert were mixed at a 1:1 molar ratio in DNA buffer with 0.5M NaCl and 25 °C over 60 min. The product was then transformed directly into GS115 cells using the EasyComp™ method from Invitrogen. Verification of plasmid recombination into the genome was achieved using two riboprimers and two "band-aid" primers. KlenTaq long and accurate DNA polymerase (14) was used at pH 9.2 for the PCR. The pPICZα "target" was prepared using two riboprimers, which amplified with an insertion gap for the trypsinogen gene. Following PCR, DpnI was added to eliminate parent DNA. The amplified products were precipitated with polyethylene glycol and washed with 70% ethanol. After resuspension in DNA buffer (10 mM Tris, 10 mM NaCl, 0.1 mM EDTA, pH 7.9) RNase A (Ambion) was added to cut the plasmid/gene at the ribosome, resulting in sticky ends on the target plasmid and the amplified trypsinogen gene. RNase A was digested by addition of proteinase K (Sigma). The prepared pPICZ target and the prepared trypsinogen gene insert were mixed at a 1:1 molar ratio in DNA buffer with 0.5 mM NaCl and annealed by heating at 75 °C for 4 min, followed by slow cooling to 25 °C over 60 min. The product was then transformed directly into XL1 Blue cells (Stratagene) and plated on low-salt LB media containing 25 µg/ml Zeocin (Invitrogen) for selection. Plasmid DNA was isolated and purified from single colonies using Maxipreps from Qiagen. The G193A and G193P mutants were constructed using the QuikChange system (Stratagene) using the pPICZα/tryspinogen plasmid as a template. Transformation and plasmid purification were done in the same manner as described for wild-type.

Expression and Purification of Trypsin Mutants in Pichia pastoris—The plasmid containing the zymogen coding region was digested with PmeI (New England Biolabs) to cut at a unique site at the 5′ AOX1 gene. The linearized plasmid was then transformed into P. pastoris GS115 cells using the EasyComp™ method from Invitrogen. Verification of plasmid recombination into the P. pastoris genome was achieved by PCR. Transformed cells were grown on YPAD plates containing 100 µg/ml Zeocin for selection. Individual colonies were screened for mutant phenotype by plating on both MDH (minimal dextrose) and MMH (minimal methanol) media and comparing the 2-day growth with mutant and wild-type standards. All selected mutants were of the mutant variety. A freshly streaked colony was used to inoculate 25 ml of buffered complex media containing glycerol in a 250-ml baffled flask at 30 °C overnight. The cells were spun down and resuspended in 100–200 ml of buffered complex media containing 2% methanol (2% MeOH) in a 1 liter baffled flask to a final A600 of 0.6–1.0 for 2–3 days at 30 °C. Every 24 h, sterile methanol was added to a final concentration of 2% to account for consumption and evaporation. Cells were pelleted by centrifugation, and the media containing trypsinogen were dialyzed/concentrated against 50 mM Tris, pH 5.0, using the Quixstand system from Amersham Biosciences with a 10-kDa hollow fiber cartridge. Trypsinogen was activated by addition of CaCl2 to 10 mM and pre-purified bovine enterokinase (Sigma), followed by incubation at 37 °C overnight. Activated trypsin was purified using an SBTI-Sepharose affinity column followed by dialysis against 1 mM HCl for storage at −80 °C.

Crystallographic data on the PPACK-inhibited forms of the thrombin mutants G193A and G193P

| Data collection | G193A | G193P |
|----------------|-------|-------|
| Wavelength (Å) | 1.281 | 1.281 |
| Space group    | P2₁,2₁,2₁ | P2₁,2₁,2₁ |
| Unit cell dimensions (Å) | a = 49.82 | a = 49.71 |
| b = 73.85 | b = 73.55 |
| c = 89.77 | c = 90.10 |
| Resolution range (Å) | 40.0–2.0 | 40.0–2.0 |
| Observations | 118809 | 231726 |
| Unique observations | 21638 | 22977 |
| Completeness | 93.8 (72.8) | 99.1 (99.1) |
| Rmerge (%) | 4.4 (15.6) | 4.9 (10.5) |
| I/σ(I) | 28.7 (7.2) | 32.1 (18.0) |
| Refinement | |
| Resolution (Å) | 40.0–2.0 | 40.0–2.0 |
| Rmerge > 0 | 0.199, 0.242 | 0.205, 0.246 |
| Reflections (working/test) | 19496/1416 | 21370/1116 |
| Protein atoms | 2366 | 2941 |
| Solvent molecules | 254 | 265 |
| r.m.s.d. bond lengths (Å) | 0.007 | 0.007 |
| r.m.s.d. angles (°) | 1.4 | 1.4 |
| r.m.s.d. B values (Å²) | 2.9/4.4 | 2.0/3.0 |
| (m.c./s.c.)² | | |
| (B) protein (Å²) | 37 | 36 |
| (B) solvent (Å²) | 48 | 47 |
| Ramachandran plot | | |
| Most favored (%) | 86.6 | 89.0 |
| Additionally allowed (%) | 12.6 | 10.1 |
| Disallowed (%) | 0.0 | 0.0 |

Additional data were collected at the Advanced Photon Source (beamline Bioсars 14-BMC, Argonne National Laboratory) and processed using the HKL2000 package (17). Crystals of both mutants were orthorhombic of space group P2₁,2₁,2₁ and contained one molecule per asymmetric unit. Crystallographic parameters are summarized in Table I. Both structures were solved by molecular replacement using the coordinates of the PPACK-bound slow form of thrombin (18) as search model and the program package CNS (19). Crystallographic refinement was carried out by simulated annealing and conjugated-gradient minimization using CNS, and model building was performed with the program O (20). The autoysis loop could not be resolved in either structure. In the G193P structure, an additional stretch of residues in the 30 loop (Ser-72 to Glu-77) was not included in the model due to weak electron density. Water molecules were added in the final stage of the refinement process. They were subject to visual inspection to check their positioning in electron density and allowed to refine freely. Water molecules with temperature factor (B-factor) higher than 80 Å² were excluded from subsequent refinement steps. Both structures contained a bound Na⁺ as wild-type, which was confirmed by valence screening with WASP (21). Coordinates of both structures have been deposited in the Protein Data Bank (accession codes Z81 for G193A and Z85 for G193P).

Determination of Individual Kinetic Rate Constants for Substrate Hydrolysis—The accepted kinetic mechanism of substrate hydrolysis by serine proteases starts with the binding of substrate S into the active site of the enzyme. The reaction is divided into several steps involving the formation of the oxyanion hole and the covalent intermediate. The constants kcat and Ks are defined as follows:

\[ \text{rate} = \frac{k_{\text{cat}} [S]}{K_s + [S]} \]

where \( k_{\text{cat}} \) is the maximum rate of the reaction and \( K_s \) is the Michaelis constant. The values of the rate constants kcat and Ks can be determined from the initial velocity data (22).

To determine the individual rate constants defin-
site of the enzyme E, with a second-order rate constant $k_1$. After formation of the enzyme-substrate complex ES, the substrate can either dissociate back into the solution with a rate constant $k_{-1}$ or become acylated to the active site Ser-195 with a rate constant $k_2$. The portion of substrate distal to the scissile bond, P', is released at this stage. The acyl intermediate $EP$ is subsequently hydrolyzed with the assistance of a water molecule to release the portion of substrate proximal to the scissile bond, P, with a rate constant $k_s$. The series of events leading to substrate hydrolysis can be depicted as follows.

$$E + S \rightleftharpoons ES \rightleftharpoons EP + P'\rightarrow E + P$$

(Eq. 1)

The Michaelis-Menten parameters $s = k_{cat}/K_m$ and $k_{cat}$ accessible to direct experimental measurements are composite functions of the individual kinetic rates in Equation 1. Specifically,

$$s = \frac{k_1 k_2}{k_{-1} + k_3}$$

(Eq. 2)

$$k_{cat} = \frac{k_1 k_3}{k_2 + k_3}$$

(Eq. 3)

The parameters have a similar form, but $s$ does not depend on the deacylation rate, whereas $k_{cat}$ does not depend on substrate diffusion into the active site and dissociation. The four independent individual rate constants defining $s$ and $k_{cat}$ can be resolved from measurements of the Michaelis-Menten parameters as a function of temperature (22). The relevant expressions are as follows:

$$s = \frac{k_1 k_2 \exp\left(-\frac{E_1 + E_2}{RT}\right)}{k_1 \exp\left(-\frac{E_1}{RT}\right) + k_3 \exp\left(-\frac{E_2}{RT}\right)}$$

(Eq. 4)

$$k_{cat} = \frac{k_1 k_2 \exp\left(-\frac{E_2}{RT}\right) + k_3 \exp\left(-\frac{E_2}{RT}\right)}{k_1 \exp\left(-\frac{E_1}{RT}\right) + k_3 \exp\left(-\frac{E_1}{RT}\right)}$$

(Eq. 5)

where $R$ is the gas constant, $T$ is the absolute temperature, and all $E$s refer to the reference temperature $T_0 = 298.15$ K. The $E$s are the Arrhenius terms defining the activation energies associated with each step of the catalytic mechanism. The eight independent parameters in Eqs. 4 and 5 can be resolved from measurements carried out as a function of temperature, provided the Arrhenius plots of the parameters $s$ and $k_{cat}$ show curvature (22). This was always the case for $s$. However, thrombin and its mutants did not show appreciable curvature in the plot of $k_{cat}$, thereby proving that the deacylation step in the catalytic conversion of FPR remains much faster than acylation over the entire temperature range studied, unlike what was observed with trypsin and its mutants. The values of $s$ and $k_{cat}$ for FPR hydrolysis were measured in the temperature range from 5 °C to 45 °C, under experimental conditions of 5 mM Tris, 0.1% polyethylene glycol, 200 mM NaCl, pH 8.0. The pH was precisely adjusted at room temperature to obtain the value of 8.0 at the desired temperature. Tris buffer has a $pK_a = 8.06$ at 25 °C and a temperature coefficient of $\Delta pK_a/\Delta T = -0.027$ (23). These properties ensured buffering over the entire temperature range examined. Unlike trypsin, thrombin is a Na⁺-activated enzyme (24), and its catalytic properties are enhanced by Na⁺ binding. The experimental conditions used in this study refer to the Na⁺-bound, fast form of thrombin (12, 22).

The values of $k_1$ determined from the temperature dependence of $s$ and $k_{cat}$ for FPR hydrolysis were checked from independent measurements of the rate of inactivation by the suicide inhibitor PPACK, which is the noncleavable analog of FPR. Progress curves of FPR hydrolysis were run in the presence of increasing concentrations of PPACK and fit to the expression

$$[P] = [P_0]\left(1 - \exp\left(-\frac{k_1 [I]}{[S]^m K_m}\right)\right)$$

(Eq. 6)

where $P$ is the $p$-nitroaniline resulting from cleavage of FPR, $S$, in the presence of PPACK, $I$, and $t$ is time.

The energetics of substrate binding and catalysis were interpreted according to the transition state theory (25, 26) using the individual rate constants determined from the temperature experiments. According to this theory, enzyme and substrate can form two complexes, ES and $ES^*$, that represent the ground and transition states, respectively. The free energy of formation of ES is given by the equilibrium expression

$$\Delta G_{ES} = RT \ln \frac{k_{-1}}{k_{cat}} = RT \ln K_m$$

(Eq. 7)

where $k$ is Boltzmann’s constant, and $h$ is Planck’s constant. The two terms differ in sign, with $\Delta G_m$ always being negative, and $\Delta G_s$ always being positive, and reflect the energetic balance of enzyme-substrate interaction before the complex is processed for catalysis. It is important to notice that both terms do not depend on the deacylation rate $k_s$, which on the other hand enters the definition of $K_m$ (see Equations 2 and 3). Hence, assessment of the properties of the ground state from measurements of $K_m$ are potentially problematic, especially when $k_s$ is not much faster than $k_2$ and when $k_2$ is comparable to $k_{-1}$ (22).

**RESULTS**

The temperature dependence of $s$ and $k_{cat}$ for the hydrolysis of FPR by thrombin and trypsin is shown in Fig. 1. Accurate measurements of both parameters were possible over the entire temperature range, enabling resolution of the relevant kinetic rate constants and activation energies. For both enzymes, the G193A mutant revealed properties closer to wild-type than the G193P mutant. Notably, thrombin always showed a linear Arrhenius plot for $k_{cat}$, implying that acylation was rate-limiting for this enzyme. On the other hand, wild-type and G193A trypsin showed a curved plot for $k_{cat}$, documenting a deacylation rate comparable to acylation. The kinetic parameters are listed in Table II.

In the case of thrombin, the wild-type binds FPR with a very fast $k_1$ (12) that approaches the diffusion-limited rate for macromolecules in aqueous solutions (27). The $k_{cat}$ is defined entirely by $k_2$ because the Arrhenius plot is linear, and $k_2 \gg k_2$ over the entire temperature range studied. Ala replacement of Gly-193 produces a notable change in $k_1$ (18-fold) and $k_2$ (4.3-fold), resulting in a 25-fold decrease in $s$ due to an increase in $K_m$ and a decrease in $k_{cat}$. Perturbing the residue in the oxyanion hole therefore changes substrate binding both in the transition state (effect on $k_{cat}$) and in the ground state (effect on $K_m$). It should be pointed out that $K_m$ for FPR hydrolysis by thrombin is not a valid measure of $K_m$ or substrate binding (Table II) and should not be used to assess effects in the ground state. The $K_m$ for FPR binding in the ground state changes from 64 nM in the wild-type to 780 nM in the G193A mutant. Hence, the effect of Ala mutation of Gly-193 on substrate binding in the ground state (12-fold reduction) is comparable to the effect on the transition state (25-fold reduction). The G193A mutation has no significant effect on the rate of substrate dissociation, $k_{-1}$, and on the Arrhenius activation energies for substrate binding, dissociation, and acylation. Notably, the value of $E_s$ for wild-type and G193A is within experimental error of the estimated 18 kcal/mol derived from ab initio QM/MM electronic calculations (28). The replacement of Gly-193 with Pro is far more deleterious on substrate hydrolysis, but qualitatively, the results are similar to those seen for the G193A mutant. There is a drastic effect on substrate binding in both the ground and transition states, with $k_1$ decreasing >800-fold and $k_2$ decreasing 28-fold. Again, the effect on $k_{-1}$ is more modest, and the resulting increase in $K_m$ is >150-fold. Unlike the Ala substitu-
Table II

Kinetic rate constants, Arrhenius activation energies, and thermodynamic parameters in the ground state (gs) and transition state (ts) for FPR hydrolysis by wild-type (wt) and mutant thrombins and trypsins

|                       | Thrombin | Trypsin |
|-----------------------|----------|---------|
|                       | wt       | G193A   | G193P   |
|                       | trypsin  | wt      | G193A   | G193P   |
| Rate constants        |          |         |         |
| $k_1$ ($\mu M^{-1}s^{-1}$) | 30 ± 3   | 2.4 ± 0.1 | 0.23 ± 0.01 | 7.0 ± 0.1 | 0.71 ± 0.02 | 0.012 ± 0.001 |
| $k_2$ ($s^{-1}$)      | 100 ± 10 | 5.5 ± 0.9 | 0.12 ± 0.02 | 9.3 ± 0.9 | 0.85 ± 0.07 | 0.0094 ± 0.0008 |
| $k_3$ ($s^{-1}$)      | 6.4 ± 0.9 | 4.2 ± 0.7 | 1.2 ± 0.2 | 2.5 ± 0.3 | 1.5 ± 0.2 | 1.0 ± 0.1 |
| $k_4$ ($s^{-1}$)      | 25 ± 1   | 5.8 ± 0.2 | 0.89 ± 0.04 | 24 ± 1   | 11 ± 1   | 3.2 ± 0.1 |
| $k_5$ ($s^{-1}$)      | ND       | ND      | ND      | 85 ± 6   | 78 ± 7   | ND       |
| $K_a$ = $k_1/k_2$ ($\mu M$) | 0.064 ± 0.006 | 0.76 ± 0.09 | 10 ± 1 | 0.27 ± 0.03 | 1.8 ± 0.2 | 110 ± 10 |
| $K_m$ ($\mu M$)       | 0.31 ± 0.03 | 1.8 ± 0.2 | 17 ± 2 | 2.2 ± 0.2 | 13 ± 1   | 450 ± 35 |
| $k_{cat}$ ($s^{-1}$)  | 25 ± 1   | 5.8 ± 0.2 | 0.89 ± 0.04 | 19 ± 1   | 9.6 ± 0.5 | 3.2 ± 0.1 |
| $s$ ($\mu M^{-1}s^{-1}$) | 81 ± 5   | 3.2 ± 0.2 | 0.052 ± 0.004 | 8.6 ± 0.5 | 0.74 ± 0.05 | 0.0071 ± 0.0004 |
| Arrhenius activation energies |          |         |         |
| $E_1$ (kcal/mol)      | 19 ± 2   | 18 ± 2   | 17 ± 2 | 14 ± 1   | 19 ± 2   | 7.1 ± 0.7 |
| $E_2$ (kcal/mol)      | 63 ± 4   | 57 ± 3   | 24 ± 1 | 49 ± 3   | 49 ± 3   | 30 ± 3  |
| $E_3$ (kcal/mol)      | 20 ± 1   | 19 ± 1   | 1.9 ± 0.3 | 22 ± 1   | 21 ± 1   | 9.1 ± 0.5 |
| $E_4$ (kcal/mol)      | ND       | ND      | ND     | 1 ± 1    | 1 ± 1    | ND     |
| Thermodynamic parameters$^c$ |          |         |         |
| $\Delta G_{gs}$ (kcal/mol) | −9.8 ± 0.1 | −8.3 ± 0.1 | −6.8 ± 0.1 | −9.0 ± 0.1 | −7.8 ± 0.1 | −5.4 ± 0.1 |
| $\Delta G_{gs}^p$ (kcal/mol) | 1.5 ± 0.1 | 3.0 ± 0.1 | 1.2 ± 0.1 | 3.6 ± 0.1 |
| $\Delta G_{gs}^p$ (kcal/mol) | 6.7 ± 0.1 | 8.6 ± 0.1 | 11.0 ± 0.1 | 8.0 ± 0.1 | 9.4 ± 0.1 | 12.2 ± 0.1 |
| $\Delta G_{gs}^p$ (kcal/mol) | 1.9 ± 0.1 | 4.3 ± 0.1 | 1.4 ± 0.1 | 4.2 ± 0.2 |

$^a$ Values refer to PPACK.

$^b$ ND, not determined because the Arrhenius plot of $k_{cat}$ is linear.

$^c$ $\Delta G$ values refer to the difference between mutant and wild-type enzyme.
tion, the presence of Pro at position 193 also influences the energetics of substrate binding and hydrolysis. Specifically, the energy barrier for substrate binding does not change, but the activation energies for substrate dissociation and especially substrate acylation change drastically. The effect on $E_a$ is particularly noticeable in Fig. 1, where the temperature dependence of log $k_{cat}$ is almost flat.

In the case of trypsin, the effects of Ala and Pro substitutions of Gly-193 mirror those observed for thrombin. The dominant effect is seen on the values of $k_1$ and $k_2$. These changes are not linked to significant energetic consequences for the Ala mutant, but they are linked to significant energetic consequences for the Pro substitution. A notable difference from thrombin is that the Arrhenius plot of $k_{cat}$ is significantly curved in the wild-type and G193A mutant of trypsin. This enables accurate measurements of $k_2$ and $E_a$. The rate of deacylation is only 3-fold faster than acylation in the wild-type and 7-fold faster than acylation in the G193A mutant. Because the rate of acylation for these enzymes is comparable to that of the thrombin enzymes, we conclude that $k_1$ values for FPR hydrolysis by trypsin must be much faster than the value obtained for wild-type trypsin. The value of $E_a$ is practically zero, signaling energetic neutrality for dissociation of the acyl enzyme. Again, this result is in striking agreement with QM/MM calculations that have reported a very small energy barrier for the break down of the acyl enzyme (28). In the case of the G193P mutant, the Arrhenius plot for $k_{cat}$ becomes linear, which could be a consequence of the drop in the acylation rate $k_a$ well below that of deacylation. As for thrombin, the Pro substitution of Gly-193 significantly lowers the activation energies for substrate dissociation and acylation.

Replacements of Gly-193 by Ala and Pro significantly affected substrate binding to the transition state, as expected from the role of the oxyanion hole in stabilizing the tetrahedral intermediate via H-bonding of the backbone O atom of the P1 residue of substrate. In addition, the replacements affected binding of substrate in the ground state, and the effect was comparable to that on the transition state. To validate the drastic effect of Gly-193 replacement on the hmollecular rate of substrate association with the enzyme, $k_1$, the rate of enzyme inactivation by the suicide inhibitor PPACK was measured in all cases. PPACK is the noncleavable analog of PPR, and its second-order rate constant $k_1$ for binding to the enzyme is expected to be in the same range as the value of $k_1$ for substrate binding. Indeed, the rate of PPACK association with the enzyme followed very closely the $k_1$ values for FPR binding (Table II), proving that mutation of Gly-193 has a direct effect on the accessibility of the active site before substrate is engaged in formation of the transition state.

The x-ray crystal structures of thrombin mutants G193A and G193P bound to PPACK were solved to high resolution (1.85 and 1.7 Å, respectively) to obtain information on the extent of structural perturbation induced by the mutations. Both structures were eventually refined to 2.0 Å to improve completeness in the higher resolution shell. The relevant crystallographic parameters are listed in Table I. The two structures display overall folds that are almost identical, having a backbone r.m.s.d. of 0.56 Å. The autolysis loop is disordered in both structures, and residues 148–149e were excluded from the models due to weak electron density. The Na$^+$ binding environment and the primary specificity pocket of both structures are practically identical with the PPACK-bound fast form FL (18). The backbone r.m.s.d. values of the two mutant structures relative to FL are 0.57 (G193A) and 0.55 (G193P), respectively. Overall, mutation of Gly-193 does not change the conformation of the enzyme, and the only appreciable effects are restricted to the site of mutation. Analysis of the solvent molecules using the program WASP (21) returned Na$^+$ specific valences of 1.13 and 1.05 valence units for the bound Na$^+$ in G193A and G193P, respectively. Because of the effect of Ala and Pro substitutions on both ground and transition state binding of substrate, it is important to assess whether proper H-bonding interactions are formed in the active site and to what extent they are perturbed. Fig. 2 shows an overlay of the FL form of thrombin 1SFQ (18) with the structures of G193A and G193P around the bound PPACK in the active site pocket. The H-bonding distances of the contacts between PPACK and thrombin are summarized in Table III. In the case of the Ala mutant, there is a slight movement of the backbone around residue 193 that brings the length of the H-bond with the backbone O atom of Arg of PPACK to 3.21 Å from 2.87 Å in the wild-type FL. The Cβ atom introduced by the mutation points away from the oxyanion hole and does not interfere with PPACK binding. The rest of the H-bonding network involving PPACK is unperturbed (Table III). In the G193P mutant, on the other hand, perturbation of the oxyanion hole is more drastic. The proline ring points away from the oxyanion hole, but its presence shifts the backbone significantly and moves the N atom to a distance of 4.45 Å from the backbone O atom of Arg of PPACK. The backbone N atom of Pro-193 has no
H-bonding capabilities, which provides a good reference model for establishing the contribution of this H-bond in the wild-type. However, the perturbation around the backbone shows that the presence of Pro at position 193 may compromise docking of substrate into the active site.

**DISCUSSION**

Gly-193 is highly conserved in serine proteases and plays a basic role in stabilizing the transition state by donating a H-bond to the backbone O atom of the P1 residue of substrate (1, 2). Although the role of this residue involves mainly its backbone N atom, there are reasons to believe that any side chain at position 193 may compromise enzyme activity and alter specificity. For example, some snake venom serine proteases carry Phe at position 193 that results in reduced activity and greater resistance to inhibition (29). Likewise, human brain trypsin is highly resistant to inhibitor inactivation and carries an Arg at position 193 (6). The crystal structure of brain trypsin shows that the side chain of Arg-193 points outward to the S2’ specificity site and does not interfere with the P1 position of substrate binding to the active site. A patient with inherited deficiency of clotting protease factor IXa was found to be homozygous for the substitution G193E (8). Expression of the G193E mutant of factor IXa revealed compromised substrate and inhibitor binding with effects on both $k_{\text{cat}}$ and $K_m$. These findings are consistent with the results on the G193A and G193P mutants of thrombin and trypsin presented here. The effect of mutating Gly-193 is seen on both $k_{\text{cat}}$ and $K_m$, as well as on $K_f$, vouching for a role of Gly-193 in substrate binding in both the ground and transition states. Interestingly, when Gly residues of the oxyanion hole of acetylcholinesterase were mutated to Ala, deleterious effects were reported on both $k_{\text{cat}}$ and $K_m$ (30). These observations suggest that mutations of Gly-193 perturb the geometry and orientation of the critical H-bond in the transition state. However, they also raise the possibility of additional effects that may result in reduced substrate binding in the ground state. The effects may be caused by steric hindrance into the active site.

Crystal structures of the thrombin mutants G193A and G193P reveal the extent of perturbation induced by the mutations. In the case of G193A, only a slight change in the length of the H-bond in the oxyanion hole is observed, whereas the G193P mutant shows abrogation of the H-bond and slight shift in the backbone geometry. It is of interest to correlate these structural changes with the energetic cost of destabilizing the enzyme-substrate complex in the ground state and in the transition state. Based on transition state theory, the enzyme E and substrate S can come together to form a complex in either the ground state (ES), with a free energy change $\Delta G_g < 0$, or in the transition state (ES$^\ddagger$), with a free energy change $\Delta G^\ddagger > 0$ (see Equations 7 and 8). The difference between $\Delta G_g$ and $\Delta G^\ddagger$ gives the activation energy associated with the chemical steps of bond making and breaking required for product formation. This term is set by the chemical nature of the reaction and is expected to be independent of perturbations of the enzyme and or substrate that do not affect the nature of the transition state (25, 26). The availability of all rate constants for the hydrolysis of FPR by thrombin and trypsin mutants makes it possible to calculate the relevant free energy terms (Table II) without making an assumption on the nature of the transition state. The cost of replacing Gly-193 with Ala in thrombin and trypsin is 1.2–1.5 kcal/mol in the ground state and 1.4–1.9 kcal/mol in the transition state (Table II). The similarity of these values suggests that the difference $\Delta G_g$ – $\Delta G^\ddagger$ is not affected by the mutation and that the perturbation of binding free energy for formation of the enzyme-substrate complex at equilibrium is transferred in its entirety to the formation of the transition state. This implies that the weakening of the H-bond contributed by residue 193 in the G193A mutant costs about 1.5 kcal/mol and that this is a lower estimate for the energetic contribution of the H-bond from Gly-193 in the oxyanion hole to substrate binding in the ground state and the transition state. The Pro replacement of Gly-193 in thrombin and trypsin costs 3.0–3.6 kcal/mol in the ground state and 4.2–4.3 kcal/mol in the transition state (Table II). Although the values are almost identical for the two enzymes, as seen for the G913A mutant, there is a significant difference between the two free energy terms. The structural perturbation induced by the G193P substitution causes a very pronounced destabilization of the enzyme-substrate complex that is 1 kcal/mol more significant in the transition state. That implies a 1 kcal/mol perturbation of the energy involved in bond making and breaking for the hydrolysis of FPR by the G193P mutants of thrombin and trypsin relative to wild-type. The Pro replacement therefore induces changes in the structure of ES$^\ddagger$ that go beyond those seen at equilibrium. Hence, the G193P mutation must cause perturbation of the enzyme beyond removal of the H-bond in the oxyanion hole. That conclusion is consistent with the crystal structure of the thrombin mutant G193P, although both the structure and energetics suggest that the additional perturbation in the Pro mutant is not drastic.

Critical assessment of the kinetic, thermodynamic, and structural consequences of replacing Gly-193 in the oxyanion hole of thrombin and trypsin leads to the conclusion that the H-bond between the N atom of Gly-193 and the O atom of the Arg of substrate contributes at least 1.5 kcal/mol and at most 3.0 kcal/mol to stabilization of the enzyme-substrate complex in the ground state or the transition state. The contribution of the H-bond of Asn-155 in the oxyanion hole of subtilisin was estimated to be 3.7 kcal/mol (10), in line with the estimate of 4 kcal/mol for the contribution of a H-bond involving a charged atom in Tyr-tRNA synthetase (26). That estimate was drawn from measurements of $k_{\text{cat}}/K_m$ only, with the contribution to the ground state being derived from the value of $K_m$ instead of $K_f$. The value determined for subtilisin is not a good gauge for the energetic contribution of Gly-193 in trypsin-like proteases, as the results presented in this study demonstrate eloquently. The difference may be due to the nature of the H-bond that involves backbone atoms in thrombin and trypsin and backbone-side chain atoms in subtilisin. Taking 1.5 kcal/mol as a lower bound for the energetic balance of H-bonding interactions involving backbone atoms in the thrombin-PPACK complex, the resulting contribution of the five H-bonds observed in the crystal structure should be 7.5 kcal/mol. The contribution of the ion pair between the guanidinium group of the Arg at P1 and the carboxylate of Asp-189 in the primary specificity pocket (Table III) is estimated to be 2.7 kcal/mol (31). Hence, the expected free energy for FPR binding to thrombin in the ground state should be at least $\sim$10 kcal/mol. Remarkably, the value determined by direct fluorescence titrations of FPR binding to the inactive thrombin mutant S195A is $\sim$10.3 kcal/mol.
We conclude that the results presented here are consistent with a number of previous studies on trypsin-like proteases, both experimental and computational, and that Gly-193 makes a crucial contribution to substrate binding in both the ground and transition states. A basic tenet of transition state theory is that the oxyanion hole interactions stabilize the transition state preferentially over the ground state to promote progression in the catalytic cycle (25, 26). The observation that Gly-193 contributes almost equally to substrate recognition in the ground and transition states is not necessarily inconsistent with the transition state hypothesis. It is possible that mutations of Gly-193 affect the ground state but not the transition state via additional mechanisms such as steric hindrance within the active site due to the bulkier side chain of Ala or Pro. In this case, the H-bonding contribution of Gly-193 in the transition state would indeed be much larger than that of the ground state, consistent with the transition state hypothesis. Inspection of the data in Table II shows that the values of $k_1$ but not $k_1$ change drastically upon mutation of Gly-193, vouching for a steric effect opposing substrate binding to the active site. However, the values of $E_1$ are not affected significantly by the mutation, except for the G193P mutant of trypsin, suggesting that steric hindrance is either small or can be removed without a large energetic penalty. Future studies on the crystal structures of the G193A and G193P mutants free of inhibitor will be necessary to resolve this point.

Acknowledgments—We are grateful to Dr. Liz Hedstrom for providing the vector of anionic rat trypsinogen. Dr. Ed Oates and Angelene Cantwell provided valuable assistance in the early stages of this study.

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Energetic and Structural Consequences of Perturbing Gly-193 in the Oxyanion Hole of Serine Proteases

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J. Biol. Chem. 2005, 280:25644-25650.
doi: 10.1074/jbc.M503499200 originally published online May 12, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503499200

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