Three Dimensional Structures of S189D Chymotrypsin and D189S Trypsin Mutants: The Effect of Polarity at Site 189 on a Protease-specific Stabilization of the Substrate-binding Site

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The crystal structure of S189D rat chymotrypsin have been determined (resolution 2.55 Å) and compared, together with D189S rat trypsin to wild-type structures to examine why these single mutations resulted in poorly active, non-specific enzymes instead of converting the specificities of trypsin and chymotrypsin into each other. Both mutants have stable structure but suffer from a surprisingly large number of serious deformations. These are restricted to the activation domain, mainly to the substrate-binding region and are larger in S189D chymotrypsin. A wild-type substrate-binding mode in the mutants is disfavored by substantial displacements of the Cys191-Cys220 disulfide and loop segments 185-195 (loop C2/D2) and 217-224 (loop E2/F2) at the specificity site. As a consequence, the substrate-binding clefts become wider and more solvent-accessible in the middle third and occluded in the lower third. Interestingly, while the Ser189 residue in D189S trypsin adopts a chymotrypsin-like conformation, the Asp189 residue in S189D chymotrypsin is turned out toward the solvent. The rearrangements in D189S trypsin are at the same sites where trypsin and trypsinogen differ and, in S189D chymotrypsin, the oxyanion hole as well as the salt-bridge between Asp194 and the N-terminal of Ile16 are missing as in chymotrypsinogen. Despite these similarities, the mutants do not have zymogen conformation. The Ser189Asp and Asp189Ser substitutions are structurally so disruptive probably because the stabilization of such a different specificity site polarities as those after the removal or introduction of a charged residue are beyond the capability of the wild-type conformation of the substrate-binding region.

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Introduction

Variation in the substrate specificity of enzymes that are structurally related and catalyze the same reaction by identical mechanism but act on different substrates is usually thought to arise from substitutions at a small number of positions rather than from a more complex restructuring. Pancreatic serine proteases, chymotrypsin, trypsin and elastase have been textbook illustrations of such enzymes: they are structurally closely related and hydrolyze peptide bonds using the same three catalytic residues His57, Asp102 and Ser195.
zymogen forms of the wild-type enzymes reveal the detailed comparisons with the active and wild-type enzymes.\textsuperscript{11,13} to six orders of magnitude less active than the two of trypsin and chymotrypsin. The mutant enzymes, this model, failed to interconvert the specificities imposed by the presence of different amino acid residues at three key positions, 189, 216 and 224.\textsuperscript{2–6}

Kinetic studies and a number of X-ray structures of inhibitor-complexed chymotrypsin and trypsin molecules showed extended enzyme–substrate interactions between the S4· · ·S1, S1′· · ·S4′ sites of the enzyme and the P4· · ·P1, P1′· · ·P4′ amino acid residues of the substrate, respectively. (The scissile bond is between the P1 and P1′ amino acid residues.)\textsuperscript{3} The S1–P1 interaction (at the specificity or primary site) exhibits a substantial side-chain specificity,\textsuperscript{8–10} imposing a 10\textsuperscript{4}–10\textsuperscript{5}-fold selectivity of trypsin substrate can be stabilized.\textsuperscript{2,3} a negative electrostatic potential, so the positive charge of a trypsin substrate can be stabilized.\textsuperscript{2,3} However, the substitutions at site 189, initiated by this model, failed to interconvert the specificities of trypsin and chymotrypsin. The mutant enzymes, D189S trypsin and S189D chymotrypsin, were two to four orders of magnitude less selective and four orders of magnitude less active than the wild-type enzymes.\textsuperscript{11,13}

An almost complete trypsin→chymotrypsin specificity conversion could be reached with the substitution of 15 amino acid residues,\textsuperscript{12,14,15} therefore, it was suggested that the determination of substrate specificity in pancreatic serine proteases is a function of an extensive set of amino acid residues, many of which are not in direct contact with the substrate.\textsuperscript{14–18} Thirteen of the substituted residues are located in two surface loops (loops C2/D2 and E2/F2) (Figure 1), in the second domain of chymotrypsin and trypsin. Further rearrangements occur in the autolysis loop of trypsin (positions 142–152) as well as, in chymotrypsin, in a loop between the D1–E1 β-barrel segments of the first domain (positions 68–82), and in the amino-terminal region from site Ile16 to Asn19.

The S1 region

The S1 region, comprising the S1 specificity site and loops C2/D2 and E2/F2 (Figure 1), is stabilized mainly by the Cys191-Cys220 disulfide, and numerous polar contacts that are internal to the region, and many of them are mediated by structural water molecules (Figure 2). The main-chain displacements show that D189S trypsin is less deformed because many amino acid residues remained in the wild-type positions in loop C2/D2, whereas in S189D chymotrypsin the wild-type conformation of the entire C2/D2 loop is lost (Table 1). The Cys191-Cys220 disulfide bridge, which is a covalent crosslink between the two sides of the S1 specificity site, is moved by 2.6–7.9 Å relative to the wild-type enzymes. It is due to the new positions of both cysteine residues in S189D chymotrypsin, while in D189S trypsin, where Cys191 retains its wild-type position, it is caused by rotations in the χ\textsubscript{1} angles and the translocation of Cys220. The displacement of the Cys191-Cys220 disulfide bond in the mutants is in sharp contrast to its highly conserved conformation among the three pancreatic serine protease types, trypsin, chymotrypsin and elastase.

The stabilization of the S1 region in the mutants is dissimilar from that in the wild-type structures. There are more interactions with external regions in S189D chymotrypsin. Eleven such interactions form in the mutant but only three in the wild-type enzyme. The most interesting difference between the S189D and wild-type chymotrypsins is the
absence of a salt-bridge in the mutant between the side-chain carboxylate group of Asp194 and the α-amino group of the N-terminal Ile16 (Figure 3), an interaction that is present in the active form of every pancreatic serine protease. The Asp194 side-chain of the mutant is stabilized in a chymotrypsinogen-like conformation (Table 2 and Figure 3). In the S1 region of D189S trypsin only the arrangement but not the number of the internal hydrogen bonds changes and the salt-bridge of the Asp194 carboxylate group with the amino group of Ile16 is intact.

The S1 specificity site

The S1 specificity site, a cleft-like structure, is distorted substantially in both mutants and for similar reasons. The middle third becomes wider and more open to the solvent due to displacements in loop C2/D2 and the shifted position of the Cys191-Cys220 disulfide. The lower third (the section around site 189) is occluded due to the translocation of sites 216–220 (loop E2/F2). In S189D chymotrypsin, the side-chain carboxylate group of aspartic acid 189 is moved by large main-chain displacements to the molecular surface, where it is completely solvent-accessible (Figure 4a). In D189S trypsin, serine 189 retains the position and conformation of the amino acid residues at this site in the wild-type enzymes.

The catalytic site

The conformation of the catalytic residues, His57, Asp102, Ser195 (the catalytic triad) and Ser214, is not influenced by the Ser189Asp and Asp189Ser substitutions (Table 2). The oxyanion hole (the peptidyl nitrogen atoms of Gly193 and Ser195) remains intact in D189S trypsin but it does not exist in S189D chymotrypsin because, adopting a chymotrypsinogen-like conformation, Gly193 is more than 4 Å from the required position (Table 2 and Figure 3).

Structural changes outside the S1 region

In D189S trypsin, significant rearrangements occur in the autolysis loop (positions 145–149). These have been discussed in an earlier publication.24

In S189D chymotrypsin, one of the two segments outside the S1 region where we observe large structural deformations is at Ile16-Gly19 on the N terminus (Table 2 and Figure 3). The Ψ torsion angle of Gly19 is rotated by ~100° relative to the active wild-type enzyme (Table 3), which moves Val17 8.0 Å apart from its wild-type position. Ile16 is disordered (not visible), it is probably out in the solvent. These deformations might be related to the absence of the salt-bridge between the α-amino group of Ile16 and the side-chain carboxylate group of Asp194 (see above).

Another segment in Ser189Asp chymotrypsin where significant (2–6 Å) displacements occur is at positions 75–79, in the surface loop D1/E1 of the first domain. This structure is known as the Ca++-binding loop because it binds a calcium ion in trypsin, the place of which is occupied by the side-chain of Glu78 in chymotrypsin. This residue is displaced in Ser189Asp chymotrypsin and an electron density, which refines better if water is

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**Figure 1.** Sequence alignment in the S1 region of pancreatic chymotrypsins and trypsins. The sequences are compared to rat chymotrypsin. Sequence identities are represented by dots. Amino acids forming the S1 specificity site are in bold capital letters and shaded. They have at least one atom within 5 Å from a P1 substrate residue. The amino acid residues in loops C2/D2 and E2/F2 are indicated by capital letters in frames. Secondary structure elements are shown above and below the sequences as: β-barrel segments (labeled C2–F2) and ⤾-turns. Chymotrypsin numbering is used.

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**Table 2.** Structural changes outside the S1 region

<table>
<thead>
<tr>
<th>Domain</th>
<th>Loop C2/D2</th>
<th>Loop E2/F2</th>
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<tr>
<td>D189S trypsin</td>
<td>Ile16-Gly19</td>
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<tr>
<td>S189D chymotrypsin</td>
<td>Ile16-Gly19</td>
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substituted for Ca$^{2+}$, suggests that a calcium ion is bound there.

**Discussion**

The X-ray structures of uncomplexed S189D rat chymotrypsin and D189S rat trypsin, a mirror image mutant pair in the chymotrypsin → trypsin and trypsin → chymotrypsin specificity conversion schemes, respectively (see Introduction), reveal stable but seriously deformed conformation in and around the S1 site. In general, chymotrypsin and trypsin molecules respond similarly to the Ser → Asp and Asp → Ser substitutions, respectively, as far as the distribution of most of the deformations is concerned, because in both mutants the changes are within the activation domain (see below) and are limited mainly to the S1 region. However, as judged from the number of sites affected and the extent of displacements, the introduction of aspartic acid caused more severe structural impacts on chymotrypsin than the removal of aspartic acid on trypsin. With the exception of Val17, Gly193 and Asp194 in S189D chymotrypsin and Ser189 and Ser190 in D189S trypsin, the conformation of the mutants in the altered regions resembles neither the active nor the zymogen form of the wild-type enzymes. Two further features of the mutants have to be mentioned in this regard: (1) D189S trypsin crystallizes in dimer$^{24}$ as α-chymotrypsin; and (2) S189D chymotrypsin appears to bind a calcium ion, which has so far been observed only in trypsin structures.

![Figure 2](image-url)
Reasons for impaired activity

Not influenced by any reordering effect (e.g., from an inhibitor), the mutant structures reveal two common causes of the poor and non-specific activity: (1) a widening and an unshielding from the solvent in the middle third; and (2) an occlusion in the lower third of the S1 specificity site. It is unlikely that a wild-type binding mode is used in these substrate-binding clefts because the stabilizing van der Waals support for the middle part of P1 residues from the Cys191-Cys220 disulfide and backbone atoms 190O–192C \( \alpha \) and 214O–221N, is missing, while steric clashes with main-chain atoms 217N–218N (S189D chymotrypsin, Figure 4(a)) and 219 (D189S trypsin) hamper a sufficiently deep penetration.

The other reasons that can cause misalignment of the scissile bond at the catalytic site and

Table 1. Structural differences measured at C\(^{\alpha} \) atoms of the S1 region between superimposed molecular models

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The abbreviation of names and the PDB accession numbers of the enzyme models that were compared are the following: chymo., (P1) phenylalanine complexed wild-type bovine chymotrypsin (6GCH); chymogen., wild-type bovine chymotrypsinogen (1CHG); tryp., BPTI complexed (P1 is lysine) wild-type rat trypsin (3TGI); trypgen., wild-type bovine trypsinogen (1TGN); S189D chymo., Ser189Asp rat chymotrypsin mutant (1KDQ); D189S tryp., Asp189Ser rat trypsin mutant (1AMH). The amino acid positions in frames are in loops C2/D2 and E2/F2. The positions that are shaded form the S1 specificity site. (For model superposition see the legend to Figure 3.)

Figure 3. The conformation and interactions of Gly193 and Asp194 in wild-type and S189D chymotrypsin and in chymotrypsinogen. Models of wild-type bovine chymotrypsin (blue), S189D rat chymotrypsin (cyan) and wild-type bovine chymotrypsinogen (magenta) are superimposed. Only the side-chains of His40 and Asp194 are shown. Zymogen-specific positions are distinguished with larger, bold italic font. For superposition, C\(^{\alpha} \) atoms at sites 44, 140, 160 and 200, which belong to the structurally conservative \( \beta \)-barrel segments, were matched. Yellow broken lines show hydrogen bonds. This image, and those in Figure 4 were generated with MIDAS software.\(^{46,47} \)

Reasons for impaired activity

Not influenced by any reordering effect (e.g., from an inhibitor), the mutant structures reveal two common causes of the poor and non-specific activity: (1) a widening and an unshielding from the solvent in the middle third; and (2) an occlusion in the lower third of the S1 specificity site. It is unlikely that a wild-type binding mode is used in these substrate-binding clefts because the stabilizing van der Waals support for the middle part of P1 residues from the Cys191-Cys220 disulfide and backbone atoms 190O–192C \( \alpha \) and 214O–221N, is missing, while steric clashes with main-chain atoms 217N–218N (S189D chymotrypsin, Figure 4(a)) and 219 (D189S trypsin) hamper a sufficiently deep penetration.

The other reasons that can cause misalignment of the scissile bond at the catalytic site and
impaired enzymatic activity are specific to the mutants and/or to the type of the substrate. One of these is the missing stabilization of positively charged trypsin substrates (P1 Lys and Arg) in the S1 site of both mutants due to the absence of hydrogen bonding partners and a negatively charged electrostatic field. This is because, in D189S trypsin, Asp189 is replaced with serine and the structural water molecules are displaced, whereas in S189D chymotrypsin Asp189 is 6.2 Å from the required position and its side-chain is turned outward, towards the solvent (Figure 4(a)). Additionally, in S189D chymotrypsin, the oxyanion hole is not formed because of a chymotrypsinogen-like conformation of Gly193.

Zymogen-like features in the S1 region of mutants

Loops C2/D2 and E2/F2, where most of the deformations are induced in the mutants, constitute the activation domain, an autonomous folding
are involved in enzyme-specific interaction networks. The conformation in these segments of the zymogen forms, which render them inactive, such as the placement of the Cys192-Cys220 disulfide and the distortions in the middle segment of the substrate-binding cleft and around amino acid residue 189, resemble the situation in S189D chymotrypsin and D189S trypsin. Other common features specific to trypsinogen and D189S trypsin are: (1) the limitation of structural deformations in the S1 site mainly to the E2/F2 loop; and (2) an induction of rearrangement towards active wild-type trypsin conformation by the addition of acetate and BPTI to D189S trypsin, and an Ile-Val dipeptide (an N-terminal analogue) and BPTI or p-guanidino benzoate to wild-type trypsinogen. Distinct structural features that are shared by chymotrypsinogen and S189D chymotrypsin are the absence of the oxyanion hole as well as the electrostatic interaction of Asp194 with His40 (Figure 3).

Although an interpretation of the zymogen-like structural features of the mutants might be that the mutations reverse the activation mechanism, as it has been suggested for D189S trypsin, it is evident from the large differences in both the main and side-chain atom positions between the mutants and zymogen enzymes (Tables 1–3) that neither mutant is in the zymogen conformation. Nonetheless, S189D chymotrypsin and D189S trypsin might represent the structure of either a frozen zymogen-like state, or an aberrant product of incomplete activation processes. Both might be the consequence of a too-high energy for wild-type conformation in the mutant S1 sites (see the next section), especially in the case of S189D chymotrypsin, where even the initial step in the transition from the zymogen to the active conformation, the formation of the salt-bridge between the Asp194 carboxylate group and the Ile16 α-amino group, cannot be accomplished.

The origin of deformations in the S1 region: an insufficient stabilization of the S1 site polarity

Similar to our findings on S189D chymotrypsin and D189S trypsin, large structural rearrangements in the regions juxtaposed within the activation domain, as well as deformations in the Ca2+–binding site (loop D1/E1) have been reported recently in a bovine trypsin-ea-antitrypsin complex. Thus, it appears that the local structure in these regions is very sensitive to perturbations. However, it is still quite unusual and surprising that a single amino acid substitution can cause such large and numerous rearrangements as those in S189D chymotrypsin and D189S trypsin.

In the C2/D2 and E2/F2 loop segments of the S1 region, where the majority of rearrangements occur in both mutants, most of the main-chain and side-chain polar atoms and structural water molecules are involved in enzyme-specific interaction networks (Figure 2), which determine the local conformation quite independently from other molecular parts. In trypsin, the side-chain carboxylate group of Asp189 is kept by this structure in a proper position for ion pair formation with a positive P1 residue and, at the same time, it is provided with a network of polar interactions that is necessary for the stabilization of such a (partially) solvent-inaccessible ionizable group and its ion pair. However, owing to its interactions, notably those with Ser190 N and Ala221a N (Figure 2(b)), the side-chain of Asp189 gains a structural role as a bridge between loops C2/D2 and E2/F2, and the two walls of the S1 site. Thus, the wild-type conformation in the S1 region of D189S trypsin is disrupted because the Asp189Ser substitution removes an integral component from the interaction system which, therefore, has to find a new arrangement. This, however, cannot be chymotrypsin-like, because of the trypsin-like amino acid sequence in loops C2/D2 and E2/F2. The role of the Asp189 side-chain carboxylate group as an organizer and stabilizer of the local conformation can also be inferred from the observations that: (1) acetate ions induce reordering in the S1 region of D189S trypsin and a more than 100-fold elevation in the trypsin activity; and (2) trypsin mutants, in which the substitutions partially fill the S1 site or relocate but do not remove Asp189, retain an essentially wild-type structure.

The necessity for a proper interaction network in the stabilization of a solvent-inaccessible ionizable group can account for the deformations found in the S1 region of the S189D chymotrypsin mutant. Modeling shows that the Asp189 residue could fit into the S1 site of chymotrypsin in a trypsin-like conformation, and even polar atoms (the peptidyl N atoms of positions 190 and 221 as well as a water molecule) are within appropriate hydrogen bonding distance. Therefore, it is probably because of the lack of stabilization of an increased S1 site polarity by a trypsin-like interaction network of loops C2/D2 and E2/F2 in chymotrypsin why the Asp189 carboxylate group of S189D chymotrypsin turns to the molecular surface and interacts with solvent molecules, thereby deforming the wild-type conformation of the S1 region.

In conclusion, the S189D chymotrypsin and D189S trypsin exemplify the structural impacts of such big changes in the local polarity as the removal or introduction of an ionizable group. The large structural rearrangements are induced probably because a balance is upset between the polarity of the S1 site and the interaction network intertwining the S1 region. Thus, the different S1 specificity of trypsin and chymotrypsin might need the complete restructuring of the immediate environment of the substrate-binding site, the C2/D2 and E2/F2 loops, in order to meet two simultaneous requirements: a very similar S1 site geometry, and a large polarity difference. Such a structural framework function of these loops in pancreatic serine proteases adds to those roles that
have been proposed: (1) a provision of enzyme specific integrity and flexibility to the activation domain, and (2) a mediation of cooperation between the S1 site and the S2, S3 subsites by, for example, defining an enzyme-specific backbone conformation to position 216. Since hydrogen bonds are very sensitive to small inaccuracies in atom positions, the reason why the complex restructuring strategy was not successful in the S1 site specificity conversions, chymotrypsin → trypsin and trypsin → elastase-1 might be the difficulty of precisely redesigning a network of 17–20 such interactions.

Materials and Methods

Expression and purification of the mutants

S189D rat chymotrypsinogen and D189S rat trypsinogen mutants were prepared from a yeast expression system. Before activation, they were further purified by hydrophobic chromatography on a phenyl-Sepharose column (Sigma Chemical Co.) as described. The active forms were obtained by an overnight activation with enterokinase (Sigma Chemical Co.) at a ratio of 20 units of enterokinase/1.0 mg of zymogen. (Note that S189D chymotrypsinogen was expressed with the propeptide of rat trypsinogen and with a Cys122Ser substitution, therefore after the activation cleavage of the Lys15-Ile16 bond, the propeptide dissociates off, and the active form did not contain the Cys1-Cys122 disulfide-linked propeptide of wild-type chymotrypsins. This zymogen form could be expressed at a higher level and allowed a trypsin contamination-free activation by enterokinase.) The activated enzymes were purified on an SBTI agarose column (Sigma Chemical Co.) as described. The purity of the enzymes was analyzed by SDS-PAGE. The enzyme concentrations were determined by BioRad protein assay.

Crystallization, data collection and structure determination of S189D chymotrypsin

S189D chymotrypsin was crystallized at room temperature by the hanging-drop, vapor-diffusion method. Crystals were obtained in a solution by mixing in a ratio of 1:1 (v/v) 1 mM HCl and 10 mM CaCl2 that contained 1.5418 Å equipped with a graphite monochromator and was run at 40 kV and 150 mA. Data processing and reduction was carried out using the programs Mosflm and Scala from the CCP4 program suite. The measured intensities were converted to structure factor amplitudes using the program TRUNCATE of the CCP4 program package. Unit cell dimensions along with other details of data collection are shown in Table 4.

The structure was solved by molecular replacement using the program AMoRe2 incorporated in the CCP4 suite. For molecular replacement calculations, a tosylated bovine α-chymotrypsin was used from the Protein Data Bank (PDB entry 2CHA) as a search model, since no structure of rat chymotrypsin is available. The atomic coordinates were modified according to the species differences and to the mutations in the recombinant S189D chymotrypsin by changing the differing amino acid residues to alanine, and by removing the first 15 N-terminal residues.

The model building was done with program O. During refinement, torsion angle dynamics, resolution-dependent weighting, bulk solvent correction and grouped B-factor refinement were used. The model has been validated by PROCHECK. The refinement statistics and parameters for the final model are given in Table 4.

The details of crystallization, data collection and structure determination of D189S trypsin (PDB entry 1AMH) have been described by Szabo et al.

Protein Data Bank accession code

The structure factors and refined coordinates have
been deposited in the Protein Data Bank with the accession code 1KDQ.

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References


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