Crystal Structure of a Complement Factor D Mutant Expressing Enhanced Catalytic Activity*

Sunghee Kim†, Sthanam V. L. Narayanan§, and John E. Volanakis‡

From the †Division of Clinical Immunology and Rheumatology, Department of Medicine and Department of Microbiology, and the §Center for Macromolecular Crystallography and School of Optometry, University of Alabama at Birmingham, Birmingham, Alabama 35294-0006

Complement factor D is a serine protease regulated by a novel mechanism that depends on conformational changes rather than cleavage of a zymogen for expression of proteolytic activity. The conformational changes are presumed to be induced by the single natural substrate, C3bB, and to result in reversible reorientation of the catalytic center and of the substrate binding site of factor D, both of which have atypical conformations. Here we report that replacement of Ser94, Thr214, and Ser215 of factor D (chymotrypsinogen numbering has been used for comparison purposes) with the corresponding residues of trypsin, Tyr, Ser, and Trp, is sufficient to induce substantially higher catalytic activity associated with a typical serine protease alignment of the catalytic triad residues His195, Asp102, and Ser94. These results provide a partial structural explanation for the low reactivity of “resting-state” factor D toward synthetic substrates.

Activation of complement leads to expression of important host defense functions and proceeds via pathways that consist of successive enzymatic amplification steps. In the alternative pathway, the first enzymatic reaction is catalyzed by factor D, a serine protease that cleaves factor B only in the context of a complex with C3b, a fragment of the third component of complement, C3 (1). Factor D is unique among serine proteases in that it requires neither enzymatic cleavage for expression of proteolytic activity nor inactivation by an inhibitor for its control. Regulation of factor D activity in blood is apparently attained by a novel mechanism that depends on conformational changes and allows for reversible expression of proteolytic activity (2, 3). The putative conformational changes are believed to be induced by the natural substrate, C3bB, and to result in reorientation of the catalytic center and of the substrate binding site of factor D, both of which have atypical structures (4). Functional support for this hypothesis has been provided by the seemingly paradoxical observation that, while the reactivity of factor D with thioester substrates and active site inhibitors is 3–4 orders of magnitude lower than that of typical serine proteases (5, 6), its proteolytic activity during complement activation is comparable to that of other complement enzymes. Obviously, esterolytic assays assess the resting-state inactive conformation of the enzyme, while complement activation assays the substrate-induced proteolytically active conformation. Structural support for the inactive resting-state conformation hypothesis has been provided by the recently determined crystal structure of factor D (4).

The two non-crystallographically related molecules, A and B, present in the triclinic unit cell of factor D have typical serine protease structural folds. However, they display distinctive orientations of the side chains of the catalytic triad residues Asp102 and His195 (chymotrypsinogen numbering1 has been used throughout this paper), while the orientation of the third member of the triad, Ser94, is similar to that of other serine proteases. In all serine proteases of known structure, the spatial relationships of these three residues are constant and essential for the formation of a functional unit responsible for catalytic activity (7–10). By contrast, in molecule A of factor D, the carboxylate of Asp102 is pointed away from His195 and is freely accessible to the solvent. In molecule B, the imidazolium of His195 is oriented away from Ser94, having assumed the energetically favored trans conformation (4). Neither of these orientations would allow expression of catalytic activity, indicating the need for a realignment of the catalytic triad residues, probably induced by the single natural substrate, C3b-complexed factor B. A possible structural explanation for the unusual disposition of Asp102/His195 in factor D is provided by the substitution of a Ser for the bulky aromatic Tyr or Trp residue usually present at position 94 of serine proteases and also of a Thr for the invariant Ser94 (Fig. 1). Both of these residues are among those identified by Blow et al. (7) as being important for shielding Asp102 from solvent molecules in chymotrypsin. In addition, a Ser residue that substitutes for the conserved aromatic Trp or Phe at position 215 may also be a determinant of the unique conformation of resting-state factor D. Indeed, in molecule B of factor D, Ser215 is positioned between Asp102 and Ser94, preventing His195 from assuming the active gauche conformation that is characteristic of serine proteases. Here we report that replacement of Ser94, Thr214, and Ser215 of factor D with the corresponding residues of trypsin, Tyr, Ser, and Trp, is sufficient to induce typical serine protease alignment of the catalytic triad residues and substantially higher catalytic activity. These results provide a structural exegesis for the unique conformation of the active center of resting-state factor D and also for its low reactivity with synthetic esters. The data also demonstrate the previously unrecognized importance of residue 94 of serine proteases for catalysis.

* This work was supported in part by National Institutes of Health Grants Al21067 and Al32949 and by Grant NAGW-813 from NASA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Division of Clinical Immunology and Rheumatology, UAB Station, THT 437, Birmingham, AL 35294-0006. Tel.: 205-934-5067; Fax: 205-934-2126.

1 For comparison purposes factor D residues are numbered similarly to chymotrypsin from 16 to 243. Nine residues present in chymotrypsin (37, 116, 117, 203, 204, 205, 206, 244, and 245) but missing from factor D are absent from the numbering scheme. Conversely, 9 residues of factor D are not present in chymotrypsin (3 residues after position 61 and one each after positions 78, 124, 129, 170, 177, and 223). These are numbered as the preceding residue with an appropriate suffix: A, B, or C.
MATERIALS AND METHODS

Construction of Wild-type (wt) and Mutant Factor D Recombinant Plasmids—The human factor D cDNA hgs11 (11) was cloned into the unique HindIII site of the eukaryotic expression vector pC7CMV (Invitrogen, San Diego, CA) as described previously (12). The S94Y mutant was constructed from the wt template by using the mutagenic oligonucleotide 5'-TGGGTGCGGAGTGATGCAGGTGG-3' (mutated nucleotides are boldface and underlined). The combination mutants S94Y/T214S, S94Y/S215W, and S94Y/T214S/S215W were constructed by using the S94Y mutant cDNA as template and the mutagenic oligonucleotides 5'-GGAGGGCAGAGAGCAGGGAG-3', 5'-GCCAGCCAGTGCCAG-3', and 5'-AACGGGCGGAGGCGG-3', respectively. Mutants were constructed according to the method of Kunkel (13). All mutations were verified by nucleotide sequencing by using the chain termination method (14). Oligonucleotides were synthesized by using a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA).

Expression and Purification of wt and Mutant Recombinant Factor D—Thirty mg of each recombinant pRc/CMV plasmid containing wt or mutant factor D cDNA were transfected into 4 x 10^5 Chinese hamster ovary (CHO) cells by electroporation at 1500 V and 25 microfarads by using a Gene Pulser apparatus (Bio-Rad). The tranfected CHO cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) supplemented with 10% heat-inactivated fetal calf serum, 2 mg glucose, 100 units/ml penicillin, and 100 mg streptomycin in 8% CO_2 at 37°C. After 48 h of culture, stably tranfected CHO cells were selected by growth for 2 weeks in the above medium supplemented with increasing concentrations (0.4-1 mM) of Genetin (418 Life Technologies, Inc.). Expression of Factor D was assayed by a solid phase enzyme-linked immunosorbent assay (ELISA) using the anti-factor D monoclonal antibody F10-11 in the solid-phase and the IgG fraction of a rabbit anti-factor D serum as detection reagent as described previously (12). For purification of Factor D, transfected CHO cells were grown in serum-free CHO medium (Life Technologies, Inc.), supplemented with 10 mg HEPES in 1-liter spinner flasks at 37°C. Typically, after 5-7 days of culture, supernatants of pooled transfecants contained approximately 1.0 mg/ml Factor D, which was purified by successive chromatography on Bio-Rex 70 (Bio-Rad) and Mono S HR 5/5 (Pharmacia Biotech Inc.) as described (15). Crystallization of the triple mutant S94Y/T214S/S215W, a single transfecant was isolated by a series of subcloning steps. The stable transfecant secreted approximately 5-10 mg Factor D in 5 days.

Hemolytic Assay for Factor D—Sheep erythrocytes carrying human C3b (EC3b) were prepared as described previously (16). The complement proteins C3 (17), Factor B (18), and properdin (19) were purified by previously described methods. Native Factor D was purified from the urine of a patient with Fanconi's syndrome as described (20). Hemolytic titrations were performed by incubating 7.5 x 10^6 EC3b with properdin (62 ng), factor B (250 ng), and appropriately diluted factor D samples in 200 µl of half-strength Veronal-buffered saline, pH 7.3, containing 2.5% dextrose, 2.5 mM MgCl_2, 10 mM EGTA, and 0.1% gelatin. The mixtures were incubated at 37°C for 30 min and then 250 µl of guinea pig serum diluted 1/40 in Veronal-buffered saline (150 mM NaCl, 5 mM Veronal, pH 7.3) containing 10 mg properdin and 0.13% gelatin (EDTA-GVB) was added. The mixtures were further incubated at 37°C for 30 min. The reaction was stopped by adding 500 µl of ice-cold EDTA-GVB, and the percentage of lysis was calculated from the absorbance of the supernatants at 413 nm and used to calculate hemolytic units/ml.

Thioester Hydrolysis by Factor D—The assay measuring the rates of hydrolysis of the thioester substrate Z-Lys-SBzl by Factor D was performed as described by Kam et al. (5). Z-Lys-SBzl was purchased from Calbiochem (San Diego, CA). Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich) was used at a concentration of 20 mM as a chromogen of thioester hydrolysis (21). Assays were conducted in microtitre wells and the rate of hydrolysis was measured as absorbance at 405 nm by using a Vmax™ kinetic microplate reader ( Molecular Devices, Menlo Park, CA). The kinetic constants were calculated from Lineweaver-Burk plots.

Crystallization and Data Collection—Diffraction quality crystals of the S94Y/T214S/S215W mutant were grown at 22°C by the vapor diffusion method by using 50 mM MES buffer and polyethylene glycol 6000 as precipitant. Crystals grew to a size of 0.3 mm x 0.3 mm x 0.5 mm in 3 days. Preliminary crystallographic analysis indicated that the crystals belonged to the hexagonal space group P3_121 with unit cell dimensions a = b = 45.4 Å, c = 175.2 Å. The calculated solvent content (22) was 43%, indicating 1 molecule/asymmetric unit. Diffraction data were collected on a Xentronics (Siemens/Nicolet, Madison, WI) area detector, using Cu-Kα radiation (40 mA, 100 kV) from a rotating anode generator. Under these conditions the crystals diffracted to a resolution of 2.0 Å. The XENGEN program package (23) was used to process the data.

Structure Determination—The structure of the S94Y/T214S/S215W mutant was solved by molecular replacement methods (24) using the atomic coordinates of native Factor D (4) as search model. The starting coordinates were modified by the removal of the side chains of the three mutated residues and also of His51, Asp102, Asp194, Ser195, Ser217, and Arg218. Coordinates of solvent atoms were also excluded in molecular replacement studies. When molecule B of the native factor D triclinic crystal was used for molecular replacement, the cross-rotation function calculations (8.0 to 4.0 Å resolution) yielded higher and cleaner peaks than when molecule A was used. Molecular B coordinates were therefore used for the molecular replacement studies. The search model was rotated and translated into the unit cell of the mutant by an R-factor search using XPLOR (25), which gave an R-factor of 38%. The positional and orientational parameters of the model were refined by using the rigid body refinement protocol in XPLOR (8.0 to 4.0 Å resolution) which gave a R-factor of 36%. The model was then further refined by a combination of simulated annealing using XPLOR and restrained least-square refinement using PROLSQ. The current refinement model includes residues 1-234 and 55 water molecules. The model has been fit by visual inspection of electron density maps (2F_o - F_i) and (F_o - F_i) computed using calculated phases (27). Table I presents the final refinement statistics.
TABLE I
Crystal and diffraction data and refinement results for the S94Y/T214S/S215W mutant factor D

| Crystal data |
|--------------|
| Space group  | p3,21 |
| Cell dimensions (Å) |
| a = 45.38 |
| b = 45.38 |
| c = 175.21 |
| α = 90.0° |
| β = 90.0° |
| γ = 120.0° |
| Molecules per asymmetric unit | 1 |
| Diffraction data |
| No. of crystals | 4 |
| Resolution (Å) | 2.0 |
| R(F) (%) | 7.63 |
| Total observations | 59,734 |
| Unique reflections (observed) | 13,899 |
| Unique reflections (possible) | 14,652 |
| (I/σ(I)) at 2.0-Å resolution | 2.12 |

Refinement results

| R (%) | 19.8 |
| R(F) | 7.5 to 2.0 |
| r.m.s. deviation from ideal values |
| Bond distances (Å) | 0.020 (0.025) |
| Angle distance (Å) | 0.035 (0.040) |
| Peptide planarity (Å) | 0.056 (0.065) |
| Planar torsion angle (degrees) | 3.1 (4.0) |
| r.m.s. deviations for isotropic temperature factors (Å²) |
| Main-chain bond | 1.01 (1.5) |
| Main-chain angle | 1.54 (2.0) |
| Side-chain bond | 1.65 (2.0) |
| Side-chain angle | 2.10 (2.5) |

* R(F) = Σ |Fo - Fc| / Σ |Fo|, where Fo and Fc are observed and calculated structure amplitudes.

** R(%) = Σ |Fo| - |Fc| / Σ |Fo|, where Fo and Fc are observed and calculated structure amplitudes.

summarizes the crystal and x-ray diffraction data and the final refinement statistics.

RESULTS

Four mutant factor D cDNAs, S94Y, S94Y/T214S, S94Y/S215W, and S94Y/T214S/S215W, were constructed for these studies. The mutant and wt factor D cDNAs were stably expressed in CHO cells, and the recombinant proteins were purified to homogeneity. The effects of the mutations on the catalytic efficiency of resting-state factor D were evaluated by an esterolytic assay using the thioester substrate Z-Lys-SBz1 (12). Results of multiple experiments are summarized in Table II. Previously reported results for the T214S, S215W, and T214S/S215W mutants of factor D (12) have been included for comparison. As shown, mutation of Ser94 to Tyr in the single S94Y mutant resulted in about 6-fold increase of the catalytic rate constant (kcat). Larger increases in kcat were observed when the S94Y mutation was combined with mutations of Thr214 to Ser and of Ser215 to Trp. The triple mutant S94Y/T214S/S215W had a kcat more than 16-fold higher than wt factor D and only 3.5-fold lower than trypsin (Table II). The enhanced kcat of all S94Y mutants and of the double T214S/S215W mutant resulted in increased overall catalytic efficiency as assessed by the kcat/Km ratio, although the Km was affected only slightly if at all.

The proteolytic activity of mutant factor D was assessed by a sensitive hemolytic assay, and the results (Table III) were in general agreement with those obtained with the esterolytic assay. The S94Y mutant had higher hemolytic activity than wt factor D, and its activity was synergistically increased in combinations with the S215W and the double T214S/S215W mutants.

Hydrolysis was measured in 0.5 M NaCl, 0.1 M Hepes, pH 7.5, containing 9% Me2SO at a substrate concentration of 0.32–1.6 mM. For trypsin 10 mM CaCl2 was added to the buffer. Ellman’s reagent 5,5’-dithio-bis-(2-nitrobenzoic acid) was used at a concentration of 20 mM as a chromogen of hydrolysis. Kinetic parameters were derived from Lineweaver-Burk plots. The values of individual parameters are the average ± S.E. of at least three experiments except for trypsin, which was tested twice. Data for T214S, S215W, and T214S/S215W are from Ref. 12.
effect on substrate binding. The second highest B-factors were observed for the Asp$^{92}$-Val$^{64}$ segment of mutant factor D. This region is flexible in molecule A, but quite rigid in molecule B of native factor D. Compared to chymotrypsin or trypsin, this segment of factor D has 3 or 4 additional residues, respectively.

Overall, the tertiary structure of the S94Y/T214S/S215W mutant is very similar to that of native factor D. The root mean square (r.m.s.) deviations between the coordinates of main chain and side chain atoms of the mutant and molecules A and B of factor D are shown in Fig. 5 (a and b, respectively). Substantial structural divergence between the mutant and both molecules of native factor D is observed in two segments. The first includes residues 169–175, which in the mutant form a flexible loop thus making the observed r.m.s. shifts an unreliable indicator of actual structural differences. Furthermore, this segment partially overlaps the region (residues 172–177) with the highest B-factors and worst real-space R-factors (Fig. 4, a and b). Excluding residues 172–177, the r.m.s. shifts between the mutant and molecule A (Fig. 5a) are 0.95 Å for main chain atoms and 1.02 Å for side-chain atoms. The corresponding values for the comparison between the mutant and molecule B are 0.86 Å and 1.59 Å. By comparison, the r.m.s. shifts between molecules A and B of native factor D (4) are 0.96 Å for main chain atoms and 1.58 Å for side chain atoms. The second segment with significant r.m.s. deviations between the mutant and both molecules of factor D includes residues 214–224. Residues 214–220 form one of the three walls that line-up the primary specificity pocket of serine proteases (30). In addition, residues 214–216 are part of the S$_1$-S$_2$ subsite$^3$ that forms a $\beta$-pleated sheet with residues P$_1$P$_2$ of the substrate (31–33). Two of these residues, Thr$^{214}$ and Ser$^{215}$, have been mutated, which probably accounts at least in part for the observed r.m.s. deviations. In addition, changes in the orientation of the side chains of Arg$^{218}$ and Arg$^{223}$ apparently contribute to the observed r.m.s. shifts. In native factor D Arg$^{218}$ forms a salt bridge with Asp$^{189}$ at the bottom of the primary specificity pocket (Fig. 6). This bridge probably contributes to the low reactivity of resting-state factor D with synthetic substrates, as it restricts access of the positively charged P$_1$Arg residue to the negative charge of Asp$^{189}$. In the mutant, Arg$^{218}$ seems to be flexible and its side chain points away from the carboxylate of Asp$^{189}$ and toward the solvent. Therefore, no salt bridge between this residue and Asp$^{189}$ can form. Instead, Arg$^{223}$ has been reoriented so that its guanidinium group is H-bonded to Asp$^{189}$ (Fig. 6). These changes are accompanied by a substantial narrowing of the primary specificity pocket of the mutant, apparently due to a movement of the segment formed by residues 214–218 (Fig. 6).

Superposition of the mutant on molecule A results in substantial r.m.s. shifts around the Ser$^{94}$–Asp$^{189}$ (Fig. 5a). Asp$^{102}$ of molecule A is pointed toward the solvent away from His$^{57}$ at the active center of factor D. In the mutant, Asp$^{102}$ is forced by the phenyl ring of Tyr$^{94}$ to assume a position similar to that observed in typical serine proteases (Fig. 6) and the entire loop joining residues Val$^{189}$ and Leu$^{193}$ has been rearranged in a way that resembles the corresponding region of trypsin. The movement of loop 89–103 is mainly responsible for the observed average shift of 5.0 Å for main chain atoms and 8.2 Å for side chain atoms of this segment (Fig. 5a). In molecule B of factor D, the orientation of Asp$^{102}$ and of the entire loop 89–103 is similar to that of trypsin, which accounts for the lack of large r.m.s. shifts between molecule B and the mutant (Fig. 5b). However, the imidazolium of His$^{57}$, which has an atypical trans orientation in molecule B of native factor B, assumes the catalytically active gauche orientation in the mutant. This movement is probably responsible for the r.m.s. shifts observed in this region of the plot shown in Fig. 5b.

Fig. 7 summarizes graphically the main structural differences between native and mutant factor D. The catalytic triad residues Asp$^{92}$, His$^{57}$, and Ser$^{215}$ of both molecules A and B present in the tridinic unit cell of native factor D have an atypical alignment inconsistent with catalysis. In molecule A, Asp$^{102}$ is turned away from His$^{57}$, having gained access to the solvent, while in molecule B His$^{57}$ is pointed away from Ser$^{215}$ and Ser$^{215}$ is occupying the space usually occupied by His$^{57}$. In contrast, in S94Y/T214S/S215W the catalytic triad residues display an orientation very similar to that of trypsin. This typical serine protease conformation of the catalytic triad probably accounts for the increased catalytic activity of the mutant compared to native factor D.

**DISCUSSION**

The present data clearly demonstrate that three residues, Ser$^{94}$, Thr$^{214}$, and Ser$^{215}$, are responsible for the atypical orientation of the catalytic triad of native factor D. Replacement of these three residues with those present in the corresponding positions of trypsin, Tyr$^{94}$, Ser$^{214}$, and Trp$^{215}$, resulted in a mutant enzyme with about 20-fold higher reactivity toward the synthetic thioester Z-Lys-SBzI than native factor D. The decreased catalytic efficiency could be accounted for by an increase in $k_{cat}$ and could be attributed to structural changes of the catalytic center. Most importantly, in contrast to native factor D, the side chains of the catalytic residues Asp$^{92}$ and His$^{57}$ of the S94Y/T214S/S215W mutant have a typical serine protease orientation very similar to that seen in trypsin (Fig. 7).

Possible lattice effects were considered before concluding that the observed trypsin-like conformational changes could be attributed directly to the mutations. The tridinic unit cell of native factor D contains two non-crystallographically related molecules, whereas the triple mutant crystallizes in a different space group with one molecule in the asymmetric unit. Therefore, different lattice forces than those exercised on either molecule A or B of native factor D should be expected to im-

---

$^3$ The nomenclature used for the individual amino acid residues (P$_1$, P$_2$, etc.) of a substrate and the corresponding subsites (S$_1$, S$_2$, etc.) of the enzyme is that of Schechter and Berger (39).
pinge on the mutant, possibly contributing to the observed realignment of key catalytic residues. However, we have recently crystallized native factor D in space group P2₁ with one molecule in the asymmetric unit. The structure of this crystal form of native factor D has been determined by a combination of isomorphous replacement and molecular replacement methods. The model has been refined to an R-factor of 18.8% by using 8.0 to 2.0 Å resolution data. The catalytic triad of this molecule has a conformation similar to that present in molecule B of the triclinic cell. Similar conformations also have been observed in inhibitor complexes of factor D. It thus seems unlikely that lattice forces are major contributors to the observed structural differences between native and triple mutant factor D.

The spatial relationships of the three catalytic residues are constant in all serine proteases of known structure and are stabilized by a network of H bonds (7, 34). Specifically, H bonds between the N<sub>d</sub>1o of His<sup>57</sup> and the O<sub>d</sub>2o of Asp<sup>102</sup>, the N<sub>e</sub>2o of His<sup>57</sup> and the O<sub>d</sub> of Ser<sup>195</sup>, and the O<sub>d</sub>2o of Asp<sup>102</sup> and O<sub>γ</sub> of Ser<sup>214</sup> that are present in typical serine proteases are also present in the mutant factor D. In contrast the orientation of the side chains of these residues in native factor D precludes the formation of these H bonds. The integrity of the H bonds and the hydrophobic nature of the environment surrounding the buried Asp<sup>102</sup> are essential for the formation of a functional unit responsible for bond formation and cleavage during catalysis (7–10).

Hence, the proposal for a substrate-induced conformational change of the active center of factor D to explain the efficient activation of the alternative complement pathway (2, 3, 5).

All three mutated residues, Ser<sup>94</sup>, Thr<sup>214</sup>, and Ser<sup>215</sup>, apparently contribute to the unusual conformation of the catalytic triad of native factor D and the resulting low reactivity toward small synthetic thioester substrates. However, Ser<sup>94</sup> seems to be the principal determinant as indicated by the larger effect of the S94Y mutation on the K<sub>cat</sub> for Z-Lys-SBzl than those obtained for the single T214S and S215W mutants (Table I). The introduction of the bulky Tyr residue at position 94 is probably responsible for forcing Asp<sup>102</sup> and His<sup>57</sup> to assume an active conformation. This is suggested by the r.m.s. deviation plot (Fig. 5a), which shows substantial structural differences in the immediate vicinity of the S94Y substitution when the mutant is superposed on molecule A of factor D.

A contribution of Thr<sup>214</sup> is clearly indicated by the synergistic gain in K<sub>cat</sub> (Table I) observed when the T214S mutation is combined with the S94Y and S215W mutations. This effect is probably due to the formation of a H bond between the side chains of Ser<sup>214</sup> and Asp<sup>102</sup>, which helps stabilize Asp<sup>102</sup>. A role for the highly conserved aromatic at position 215 of serine proteases in catalysis has never been probed before. This residue is part of the S<sub>1</sub>-S<sub>3</sub> subsite in trypsin (33), which forms a β-pleated sheet with residues P<sub>1</sub>-P<sub>3</sub> of the substrate. In trypsin, Trp<sup>215</sup> is involved in the formation of a hydrophobic cluster.
Fig. 6. Comparison of the primary specificity pockets of the S94Y/T214S/S215W mutant (black) and molecule B of native factor D (red). The catalytic residue Ser195 is shown at the top right corner and Asp189, which in trypsin-like serine proteases forms a salt bridge with the side chain of the P1 Arg, is shown on the right side of the pocket. The guanidino group of Arg218 is salt-bridged to Asp189 in the native structure, but it is oriented away from Asp189 in the mutant. This shift is compensated by a reorientation of Arg223, the side chain of which is H-bonded to Asp189 in the mutant. The largest structural differences between the two structures are due to narrowing of the pocket of the mutant.

Fig. 7. Active site regions of molecule A (MOLA), and molecule B (MOLB) of native factor D, bovine trypsin, and factor D S94Y/T214S/S215W mutant (STS). The models were generated using the Ribbons program (38). Green is used for carbon, blue for nitrogen, and red for oxygen. The backbone is represented as a tube, and bonds joining the side-chain atoms Cβ onward are shown as cylinders. Each model shows the orientation of the catalytic triad and of residues in the immediate vicinity. Bovine trypsin coordinates were obtained from the Brookhaven Protein Data Bank.
that also includes Tyr^172, a structural determinant of substrate specificity (29). However, in the S94Y/T214S/S215W mutant loop 172–177 is flexible, precluding a hydrophobic interaction between His^172 and Trp^215. Instead, Trp^215 is oriented in a way favoring hydrophobic interactions with His^57 (Fig. 7). Regardless of its precise structural contribution, the S215W mutation had a significant effect on catalytic efficiency against Z-Lys-SBzl (Table II).

An additional interesting structural difference between S94Y/T214S/S215W and native factor D involves Arg^218. In both molecules of factor D, the guanidinium group of this residue forms a salt bridge with the carboxyl of Asp^189 (4). In trypsin and presumably also in all trypsin-like serine proteases, the carboxyl of Asp^189 forms a salt bridge with the side chain of the P1 Arg or Lys residue of the substrate (35). This ionic interaction plays a major role in positioning the scissile bond of the substrate for hydrolysis. Thus, the Arg^218-Asp^189 interaction between Arg^218 and negatively charged residue(s) indicates a linkage among the conformations of these elements (36). These findings were interpreted to indicate a direct interaction between Arg^218 and determinants on the C3bB complex, the natural substrate of factor D.

The present finding that the realignment of the catalytic triad residues induced by the S94Y/T214S/S215W mutations is associated with a reorientation of Arg^218 away from Asp^189 indicates a linkage among the conformations of these elements of the active center. It thus seems possible that the proposed interaction between Arg^218 and negatively charged residue(s) on C3bB not only makes Asp^189 available to the P1 Arg^234 of factor B, but also contributes to the realignment of the catalytic triad residues. Obviously, additional conformational changes particularly of the substrate binding pocket are necessary for expression of efficient proteolytic activity. These changes were not induced by the mutation of the three residues investigated in the present study, as indicated by the geometry of the primary specificity pocket (Fig. 6) and the high K_m of the triple mutant (Table II).