Mutational Analysis of the Substrate Binding Site of Human Complement Factor D†

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ABSTRACT: Complement factor D is a serine protease with a single natural substrate, C3b-complexed factor B, and very low catalytic activity against synthetic esters. The recently solved X-ray crystal structure of factor D has demonstrated certain key differences from other serine proteases in the conformation of residues of the catalytic triad and the substrate-binding regions. To investigate possible contributions of unique amino acid substitutions to these distinct structural and functional features of factor D, we constructed a series of mutants by substituting trypsin substrate-binding residues for the corresponding factor D residues. Wild-type and seven mutant factor D cDNAs were expressed stably in Chinese hamster ovary cells, and the recombinant proteins were purified from culture supernatants and assayed by hemolytic, proteolytic, and esterolytic assays. The combined results indicate that residues Thr-198, Ser-199, Arg-202, and perhaps also Val-203 provide determinants for substrate binding and catalysis. The data also provide additional support for the hypothesis that the proteolytically active conformation of the active center of factor D is induced by its substrate, C3bB.

Factor D is a serum serine protease necessary for the formation of the C3-convertase in the alternative pathway of complement activation. It accomplishes its function by cleaving a single arginyl−lysyl bond of factor B in the context of the Mg^{2+}-dependent complex, C3bB (Lesavre et al., 1979). Factor D is irreversibly inhibited by diisopropyl fluorophosphate (Fearon et al., 1974) and displays a high degree of amino acid sequence homology with other serine proteases, particularly rat mast cell protease, human neutrophil and porcine pancreas elastase, and bovine chymotrypsin and trypsin (Niemann et al., 1984b). Recent X-ray diffraction studies revealed that the general structural fold of factor D is very similar to that of other serine proteases of known structure (Narayana et al., 1994). However, compared to classic serine proteases, factor D has very low catalytic activity against synthetic ester substrates (Kam et al., 1987), reacts only moderately with inhibitors of serine proteases (Kam et al., 1992), and has a single known natural substrate, C3b-bound factor B. In addition, factor D is secreted and circulates in blood in its enzymatically active form devoid of an activation peptide, but also devoid of proteolytic activity against uncomplexed factor B. The structural determinants of these unique functional characteristics are unknown, but it has been proposed that a conformational change of the catalytic center of factor D is induced by the natural substrate C3bB, resulting in the expression of the full catalytic potential of the enzyme (Kam et al., 1987).

Detailed structural and functional studies of other serine proteases have indicated that three regions corresponding to amino acid residues 177−183, 198−205, and 210−213 of factor D (Figure 1) form the walls of the primary specificity pocket (Cohen et al., 1981; Hedstrom et al., 1992). Additional X-ray crystallographic studies of complexes between trypsin (Ruhlmann et al., 1973), chymotrypsin (Segal et al., 1971), and subtilisin (Robertus et al., 1972) and active-site inhibitors of these enzymes have indicated that the region corresponding to factor D residues 198−200 forms a short antiparallel β-sheet with residues of the inhibitor corresponding to the P_1−P_3[1] subsites of the substrate. Residues of substrate-binding regions have been conserved in factor D except for a few key substitutions which could be significant in determining its low esterolytic activity and extremely limited substrate specificity. Comparison of the 2.0-Å crystal structure of factor D to those of other serine proteases indicates unique conformations of catalytic and substrate-binding residues which would preclude expression of catalytic activity unless a realignment is induced by a conformational change (Narayana et al., 1994). In order to investigate the possible participation of unique factor D residues in substrate binding and catalysis, we have constructed a series of mutants by substituting trypsin for factor D residues. Expression and functional assessment of these mutants has allowed for an initial description of the substrate-binding site of factor D.

MATERIALS AND METHODS

Construction of Wild-Type (wt)[2] and Mutant Factor D Recombinant Plasmids. The human factor D cDNA clone hg31 was obtained from Dr. R. Tyler White (California Biotechnology Inc., Mountain View, CA). hg31 encodes the 228 amino acids of mature factor D preceded by an activation

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[1] 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 (1967).

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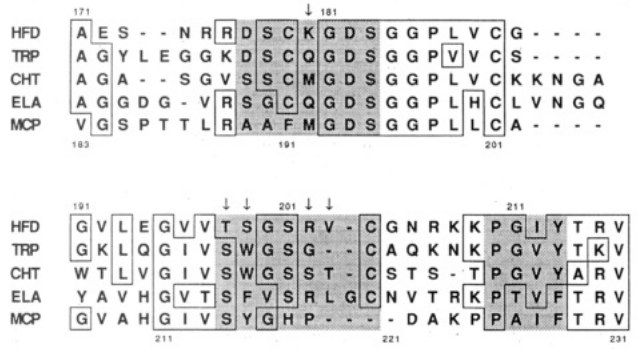


FIGURE 1: Alignment of partial amino acid sequences of selected serine proteases. Residues participating in the formation of the primary and extended substrate binding sites (Hedstrom et al., 1992) are shaded, and conserved residues are boxed. Numbers at the top are for resiues of the human factor D sequence while numbers at the bottom are for chymotrypsinogen residues. Arrows indicate residues subjected to site-directed mutagenesis. Abbreviations: HFD, human factor D; TRP, bovine trypsin; CHT, bovine chymotrypsin; ELA, porcine elastase; MCP, rat mast cell protease. Sequence data are from Greer (1990) except for the HFD sequence, which is taken from White et al. (1992).

peptide of 7 amino acids and an 18-residue-long chimeric signal peptide encoded by 21 nucleotides derived from the mouse factor D cDNA and 33 nucleotides of the human factor D cDNA (White et al., 1992). The insert of clone hg31 was isolated and subcloned into the unique *Hin*dIII site of the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA). The orientation of the insert with respect to the cytomegalovirus promoter of the vector was determined by digestion of the recombinant plasmid with *Bam*HI, followed by agarose gel electrophoresis. The M13 origin of replication of the vector facilitated the production of single-stranded DNA, which was used for sequencing and also for site-directed mutagenesis. Mutants were constructed by using mutagenic oligonucleotides according to the method of Zoller and Smith (1983) as modified by Kunkel (1985). All mutations were verified by nucleotide sequencing using the chain-termination method (Sanger et al., 1977). The sequences of the mutagenic oligonucleotides used and the corresponding changes in the amino acid sequence of factor D are presented in Table 1. All synthetic 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 (r-Factor D). Twenty micrograms of each recombinant pRc/CMV plasmid containing wt or mutant factor D cDNA was transfected into 4×10^6 Chinese hamster ovary (CHO) cells by electroporation at 1500 V, 25 μF, by using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA). The transfected CHO cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in 8% CO_2 at 37 °C. After 48 h of culture, stably transfected CHO cells were selected by growth for 2 weeks in the above medium supplemented with increasing concentrations (0.4−1 mg/mL) of geneticin-418 (GIBCO/BRL, Gaithersburg, MD). Expression of r-factor D was assessed by a solid-phase enzyme-linked immunosorbent assay (ELISA) described below. For purification of r-factor D, transfected CHO cells were grown in serum-free CHO medium (GIBCO/BRL), supplemented with 10 mM Hepes in 1-L spinner flasks at 37 °C. Typically, culture supernatants of pooled transfectants yielded approximately 1 μg/mL r-factor D, which was purified by successive chromatography on Bio-Rex 70 (Bio-Rad) and Mono S HR 5/5 (Pharmacia Biotech Inc., Piscataway, NJ).

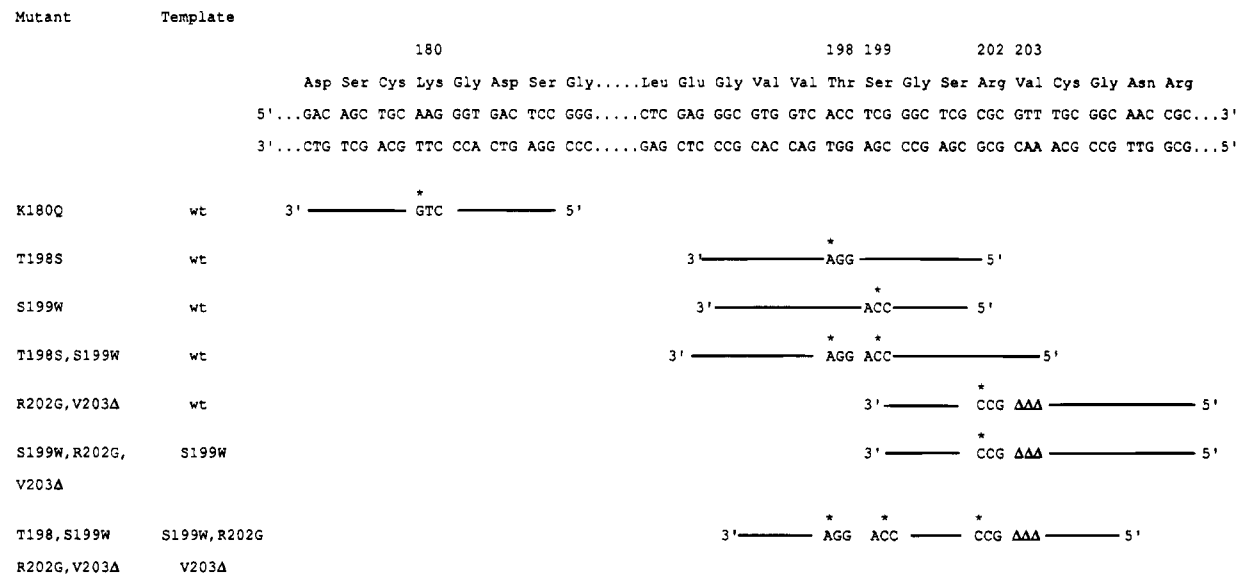
ELISA for Factor D. The concentration of factor D was measured by a solid-phase sandwich-type ELISA adapted from a previously described radioimmunometric assay (Barnum et al., 1984). An affinity-purified anti-factor D monoclonal antibody, FD10-1, was used at 25 μg/mL to coat the wells of 96-well microtiter plates, and caprylic acid-purified rabbit anti-factor D IgG, at 5 μg/mL, was used as the detection reagent. The assay was developed with affinity-purified peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) followed by substrate [0.04% (w/v) *o*-phenylenediamine dihydrochloride/0.012% (v/v) H_2O_2 in 0.1 M citrate buffer, pH 4.5]. The reaction was stopped by addition of 2 N H_2SO_4, and color development was measured at 492 nm by using an ELISA plate reader (Titertek Multiscan, Flow Laboratories, Rockville, MD). The concentration of factor D was estimated from a standard curve constructed with native factor D purified from the urine of a patient with Fanconi's syndrome (Volanakis & Macon, 1987). The concentration of the native factor D was determined from absorbance at 280 nm using an extinction coefficient, $E^{1\%}_{1cm} = 16$, which corresponds to values determined by amino acid analysis (Volanakis & Macon, 1987).

Hemolytic Assay for Factor D. Sheep erythrocytes carrying human C3b (EC3b) were prepared as described previously (Ueda et al., 1987). C3 (Gresham et al., 1986) and properdin (Pangburn, 1989) were purified by previously described methods. Hemolytic titrations were performed by incubating 7.5×10^6 EC3b with properdin (62 ng), factor B (250 ng), and dilutions of the factor D samples in a total volume of 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, pH 7.3, containing 10 mM EDTA and 0.1% gelatin (EDTA-GVB) was added. The mixtures were further incubated at 37 °C for 30 min. The reaction was stopped by addition of 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 per milliliter.

Factor B Cleavage Assay. The factor B-cleaving activity of wt and mutant r-factor D was assessed as follows: Factor B (1 μg) was incubated with 100 ng of factor D in the presence of cobra venom factor (CoVF) (1.5 μg) or in the absence of CoVF at 37 °C for 60 min in a total volume of 25 μL of 75 mM NaCl, 1 mM $MgCl_2$, and 25 mM Tris buffer, pH 7.3. Reactions were stopped by addition of nonreducing SDS−polyacrylamide gel electrophoresis (PAGE) sample buffer followed by heat denaturation at 100 °C for 10 min. Proteins were resolved by electrophoresis in a 10% SDS−PAGE as described by Laemmli (1970) and visualized

[2] Abbreviations: wt, wild type; r-factor D, recombinant factor D; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; EC3b, sheep erythrocytes carrying human C3b; EDTA-GVB, veronal-buffered saline, pH 7.3, containing 10 mM EDTA and 0.1% gelatin; CoVF, cobra venom factor; Z, benzyloxycarbonyl; SBzl, thiobenzyl.

Table 1: Template cDNAs and Mutagenic Oligonucleotides Used in Constructing Factor D Mutants[a]



[a] The nucleotide and deduced amino acid sequences of the wt factor D cDNA are shown. Residue numbers of mutated amino acids are indicated. Lines indicate identity with the nucleotide sequence of the noncoding strand of factor D cDNA. Asterisks mark nucleotide mismatches. Δ denotes amino acid or nucleotide residue deletion.

by staining with Coomassie blue. Factor B was purified from outdated human plasma as described previously (Niemann et al., 1980). CoVF was purchased from Natural Source (Houston, TX).

Hydrolysis of Thioesters by Factor D. Assays measuring the rates of hydrolysis of thioester substrates by factor D were performed as described by Kam et al. (1987). Z-Arg-SBzl was kindly provided by Dr. Chi-Min Kam (Georgia Institute of Technology, Atlanta, GA) and Z-Lys-Arg-SBzl by Dr. David Shrout (BioCryst Pharmaceuticals, Inc., Birmingham, AL). Z-Lys-SBzl was purchased from Calbiochem (San Diego, CA). Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, WI), was used at a concentration of 20 mM as a chromogen of thioester hydrolysis (Habeeb, 1976). The assays were conducted in microtiter wells, and the rate of hydrolysis was measured as absorbance at 405 nm by using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA). Kinetic constants were determined from the slopes of Lineweaver–Burk plots. Because of limited solubility of the substrates, in most cases the plot went through the origin. Therefore, only the ratio k_{cat}/K_m but not the individual constants k_{cat} and K_m could be accurately determined.

Statistics. The hemolytic and esterolytic activities of wt and mutant r-factor D were compared to those of native factor D by using an unpaired two-tailed Student's *t*-test.

RESULTS

Stable CHO cell lines secreting wt and all seven trypsin-like factor D mutants (Table 1) were readily established. It has been reported (White et al., 1992) that CHO cells transfected with wt hg31 cDNA secreted active factor D lacking an activation peptide. By contrast, expression of the same cDNA in insect cells by using a baculovirus expression system resulted in secretion of trypsin-activatable profactor D, displaying a heptapeptide extension of the NH_2 terminus of the mature enzyme (Yamauchi et al., 1994). The CHO cell lines developed in this study secreted predominantly active factor D and only small amounts of the zymogen form. The latter could be differentiated from active factor D by SDS–PAGE and Western blotting and could be converted to hemolytically active factor D by trypsin as reported previously (Yamauchi et al., 1994). The two forms of factor D were easily separated from each other by successive chromatography on Bio-Rex 70 and Mono S. This allowed the purification to homogeneity of wt and all species of mutant r-factor D in active form. As shown in Figure 2, all species of r-factor D had the same SDS–PAGE mobility as native factor D. Furthermore, all species of r-factor D were reactive with both rabbit anti-factor D antibodies and the monoclonal antibody FD10-1 (Niemann et al., 1984a).

The functional activity of wt and mutant r-factor D was initially evaluated by a sensitive hemolytic assay, which provides a quantitative measure of the proteolytic activity of factor D against its natural substrate, C3bB. The results of three independent experiments are summarized in Table 2. As shown, wt r-factor D and the single-residue mutants T198S and S199W expressed specific hemolytic activities similar to that of native factor D. The double mutant T198S,-S199W and the K180Q mutant had reproducibly higher activity than native factor D. However, their respective differences from native factor D did not reach statistical significance. Substitution of Gly for Arg-202 in combination with deletion of Val-203 caused a pronounced decrease in hemolytic activity. As shown, the specific hemolytic activity of all three mutants carrying these combined mutations was more than 2 orders of magnitude lower than that of native factor D.

As expected, the results of the hemolytic assays were in good agreement with those of the factor B cleavage assay (Figure 3). CoVF was substituted for C3b in this assay because it forms a more stable complex with factor B (Cooper, 1973). All mutants that expressed hemolytic

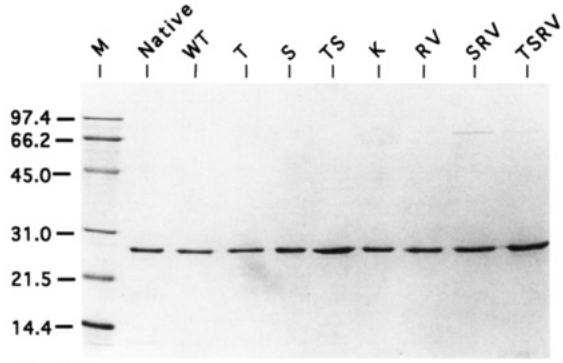


FIGURE 2: SDS−PAGE analysis of purified native and r-factor D. Approximately 1 μg of native factor D or r-factor D was subjected to SDS−PAGE in a 12% gel and stained with Coomassie blue as described by Laemmli (1970). Abbreviations: M, molecular size markers; WT, wild type; T, T198S; S, S199W; K, K180Q; R, R202G; V, V203Δ. The molecular mass of the markers in kilodaltons is shown on the left.

Table 2: Hemolytic Activity of Wild-Type and Mutant Recombinant Factor D

factor D	specific hemolytic activity[a] (units/μg)
native	24 218 ± 1479
wild type	26 142 ± 2071
K180Q	33 392 ± 2802
T198S	22 838 ± 622
S199W	21 953 ± 430
T198S,S199W	29 835 ± 2750
R202G,V203Δ	157 ± 8[b]
S199W,R202G,V203Δ	45 ± 2[b]
T198S,S199W,R202G,V203Δ	43 ± 4[b]

[a] EC3b were incubated with properdin, factor B, and dilutions of factor D at 37 °C for 30 min. Guinea pig serum diluted in EDTA-GVB was added, and the mixtures were incubated at 37 °C for 30 min. The percentage of lysis was calculated from the absorbance of supernatants at 413 nm and used to calculate the hemolytic units per milliliter. Factor D concentration was measured by ELISA and was used to calculate specific activity in units per microgram. The mean ± SE values from three independent experiments are shown. Other experimental details are given in the Materials and Methods section. [b] $p = 0.0001$, by comparison to native factor D.

activity in the same range as native factor D cleaved factor B into Bb and Ba only in the presence of CoVF. In all cases factor B was essentially completely cleaved at the end of the 1-h incubation, a fact that precluded quantitative comparisons among the different factor D species. All three R202G,V203Δ mutants failed to cleave factor B, which is consistent with the virtual loss of hemolytic activity of these mutants (Table 2).

The enzymatic activity of the r-factor D mutants was further evaluated by esterolytic assays using a dipeptide and two amino acid thioesters as substrates. Of these, Z-Arg-SBzl and Z-Lys-Arg-SBzl, which correspond to the cleavage site of factor B, were shown previously to be reactive with factor D (Kam et al., 1987). The third thioester Z-Lys-SBzl had not been tested before, but it was included in the present study because it is a sensitive substrate for trypsin and other trypsin-like serine proteases (Green & Shwa, 1979). The kinetic parameters for the hydrolysis of these substrates by bovine trypsin were also determined for comparison purposes. Results of multiple experiments summarized in Table 3 demonstrated interesting differences among the various mutants. Noteworthy observations include the increased reactivities of the S199W and the T198S,S199W mutants

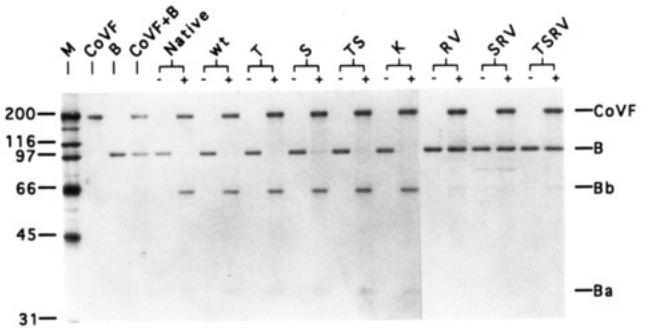


FIGURE 3: Factor B cleavage assay. SDS−PAGE analysis of reactions of factor B with native factor D and r-factor D. The samples were not reduced, and the 10% gels were stained with Coomassie blue. Abbreviations: M, molecular size markers of the indicated molecular mass in kilodaltons; CoVF, cobra venom factor; B, factor B. Ba and Bb are the two fragments generated by the factor D-catalyzed cleavage of factor B. Designations of other lanes refer to the species of factor D used. Abbreviations: wt, wild type; T, T198S; S, S199W; K, K180Q; R, R202G; V, V203Δ. Each factor D species was incubated with factor B either in the absence (−) or in the presence (+) of CoVF. Other experimental details are given in the text.

toward the Z-Arg-thioester, the increased reactivity of the T198S,S199W and the R202G,V203Δ double mutants toward the Z-Lys-thioester, the moderate increase in the overall reactivity of the K180Q mutant, and the increased reactivity of the R202G,V203Δ mutant toward the Z-Arg-thioester. Curiously, combinations of the R202G,V203Δ mutations with the S199W mutation, which by itself also caused increased rates of hydrolysis of the Z-Arg-thioester, resulted in significantly decreased activity toward this substrate.

In Figure 4, the reactivities of individual mutant r-factor D toward the three thioesters are compared to each other and to their hemolytic activities. To compensate for differences in assay sensitivities and in units of measurement, all values have been normalized relative to those of native factor D. Two interesting observations emerge from this analysis. First, the two double mutants, T198S,S199W and R202G,-V203Δ, demonstrate increased reactivity toward single amino acid substrates, particularly the Lys rather than the Arg thioester. Second, a remarkable dissociation between hemolytic and esterolytic activities was caused by the R202G,V203Δ mutation. All three species of r-factor D that contain this mutation have virtually no proteolytic activity, yet they express substantial and, in some cases, increased reactivity toward thioester substrates.

DISCUSSION

The results of this study indicate that residues 198−203 of factor D contribute to substrate binding and catalysis. The data also provide additional support for the hypothesis that the proteolytically active conformation of factor D is induced by its only known substrate, C3b-complexed factor B (Kam et al., 1987). This hypothesis was proposed to reconcile the discrepancy between the extremely low reactivity of factor D toward thioester substrates and its apprently high proteolytic efficiency during activation of the alternative complement pathway. The latter is deduced from the kinetics of alternative pathway activation in blood, which is comparable to that of the classical pathway. In the classical pathway, C1s, the functional homologue of factor D, circulates as a

Table 3: Hydrolysis of Synthetic Thioester Substrates by Wild-Type and Mutant Recombinant Factor D

factor D	k_{cat}/K_m, M^{-1} s^{-1} (n)[a]		
	Z-Lys-Arg-SBzl	Z-Arg-SBzl	Z-Lys-SBzl
native	386 ± 14 (2)	191 ± 18 (3)	349 ± 17 (5)
wild type	382 ± 26 (3)	191 ± 28 (5)	336 ± 23 (4)
K180Q	649 ± 12 (2)[e]	321 ± 7 (2)[d]	391 ± 32 (3)
T198S	342 ± 5 (2)	99 ± 9 (3)[d]	326 ± 24 (3)
S199W	265 ± 28 (2)	406 ± 46 (3)[d]	394 ± 18 (3)[c]
T198S,S199W	559 ± 13 (2)[d]	482 ± 6 (2)[f]	1122 ± 97 (3)[g]
R202G,V203Δ	198 ± 18 (2)[d]	317 ± 11 (4)[e]	873 ± 74 (4)[g]
S199W,R202G,V203Δ	55 ± 10 (2)[e]	125 ± 14 (3)[c]	274 ± 23 (4)
T198S,S199W,R202G,V203Δ	62 ± 5 (2)[e]	109 ± 10 (3)[c]	525 ± 23 (3)[g]
trypsin[b]	1×10^6	3.6×10^6	1.3×10^6

[a] k_{cat}/K_m values were derived from Lineweaver−Burk plots. Results are the mean ± SE values of the indicated number (n) of independent experiments. Hydrolysis of substrates by factor D was measured in 0.5 M NaCl−0.1 M Hepes buffer, pH 7.5, containing 9.8% Me_2SO. Substrate concentrations were 0.16−0.8 mM. Z, benzyloxycarbonyl; SBzl, thiobenzyl. [b] Hydrolysis of substrates by trypsin was measured in 10 mM $CaCl_2$−0.1 M Hepes buffer, pH 7.5, containing 9.8% Me_2SO. Substrate concentration was 8−80 μM. [c] $p < 0.05$. [d] $p = 0.01$. [e] $p < 0.005$. [f] $p = 0.001$. [g] $p = 0.001$. All comparisons are to native factor D.

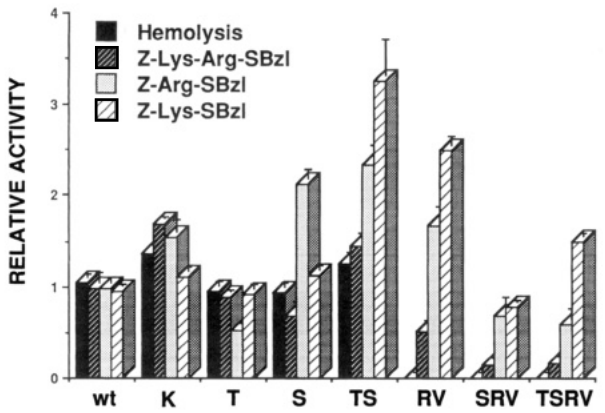


FIGURE 4: Comparison of the hemolytic and esterolytic activities of wt and mutant r-factor D. Hemolytic and esterolytic activity values of native factor D have been used to normalize the corresponding values of each r-factor D species.

zymogen which once activated expresses esterolytic activity comparable to that of trypsin (Kam et al., 1987) and proteolytic activity against uncomplexed C4 and C2, its natural substrates. In contrast, factor D has no structural zymogen in the blood and expresses low esterolytic activity and no proteolytic activity against native proteins including uncomplexed factor B, hence, the proposal for a substrate-induced active conformation. Support for this hypothesis was recently provided by the crystal structure of factor D (Narayana et al., 1994).

The structure of factor D was solved at 2.0-Å resolution by a combination of multiple isomorphous and molecular replacement methods (Narayana et al., 1994). Two non-crystallographically related molecules, A and B, were present in the triclinic unit cell. Their overall structures were similar to each other and to those of other serine proteases (Fehlhammer et al., 1977; Blevins & Tulinsky, 1985; Bode et al., 1983, 1989; Blow et al., 1969). However, the two molecular forms of factor D differed from each other and from other serine proteases significantly with respect to the conformational and spatial relationships of the side chains of certain catalytic and substrate-binding residues. The most important of these differences are shown schematically in Figure 5, which compares the active site of trypsin to the corresponding regions of molecules A and B of factor D. To facilitate comparisons, factor D numbering has been used for all three structures in Figure 5. However, for cross-referencing purposes, throughout the following discussion, the corresponding chymotrypsinogen numbers are given in parentheses.

In all serine proteases of known structure, the catalytic triad residues, which correspond to factor D residues Asp-89(*102*), His-41(*57*), and Ser-183(*195*), display similar spatial relations as they do in trypsin (Figure 5). The orientation of the side chains of these residues is maintained by a network of hydrogen bonds including those between Nδ1 of His-41(*57*) and Oδ2 of Asp-89(*102*), Nε2 of His-41(*57*) and Oδ of Ser-183(*195*), and Oδ2 of Asp-89(*102*) and Oγ of Ser-198(*214*) (Blow et al., 1969; Stroud et al., 1971). In factor D the relative positions of the side chains of these residues are markedly different, precluding the formation of most of these hydrogen bonds. In molecule A of factor D the carboxylate group of Asp-89(*102*) is pointed away from the active site, thus failing to stabilize the imidazole ring of His-41(*57*). In molecule B, Asp-89(*102*) is in the same position as in trypsin (Figure 5), but the side chain of His-41(*57*) assumes the energetically favored *trans* conformation. The space occupied in trypsin by the side chain of His-41-(*57*) is filled by Ser-199(*215*). Thus, in both molecular forms of factor D, the orientation of the catalytic residues would not allow expression of catalytic activity, indicating the need for a realignment effected through conformational change and probably induced by the substrate.

The unique disposition of the catalytic residues of factor D could be attributed, at least in part, to substitution of two residues highly conserved in most serine proteases. As shown in Figure 1, in most serine proteases a Ser is present at the position corresponding to Thr-198(*214*) of factor D, and a Trp, Phe, or Tyr is present at the residue corresponding to Ser-199(*215*) of factor D. In serine proteases of known structure, these two residues are part of the S_1-S_3 subsite participating in the formation of a β-pleated sheet through hydrogen bonds with residues P_1-P_3 of the substrate (Ruhlmann et al., 1973; Segal et al., 1971; Robertus et al., 1972). These interactions are important for the proper positioning of the side chain of the P_1 residue of the substrate into the primary specificity pocket, and they also contribute to the stabilization of the transition state. In addition, the side chain of Ser-198(*214*) forms a hydrogen bond with Asp-89(*102*), thus contributing to its correct orientation away from the solvent. In factor D, the hydroxyl group of Thr-198-(*214*) is far from Asp-89(*102*) and thus cannot form a

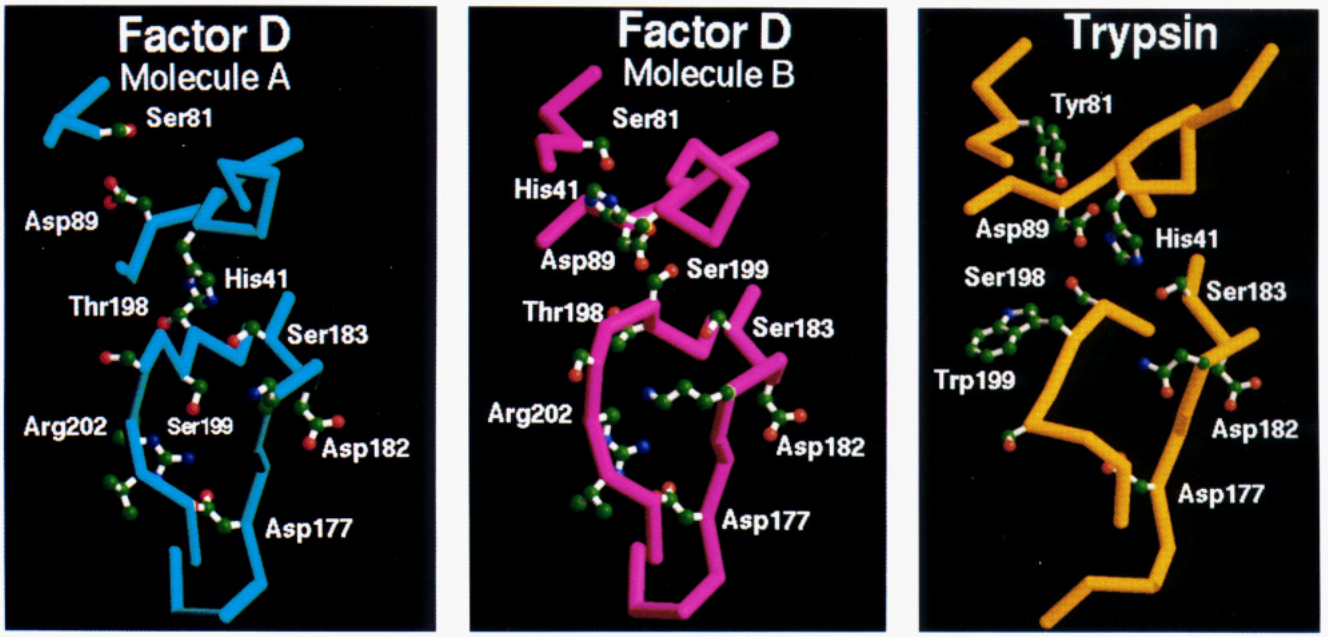


FIGURE 5: Cα models of the active centers of the two molecular forms of factor D and of trypsin. The side chains of the residues forming the catalytic triad and of substrate binding residues are shown. Factor D numbering has been used for all three molecules. Correspondence to chymotrypsinogen numbering (in parentheses) is as follows: His-41(*57*), Asp-89(*102*), Asp-177(*189*), Lys-180(*192*), Asp-182(*194*), Ser-183(*195*), Thr-198(*214*), Ser-199(*215*), and Arg-202(*217*).

hydrogen bond with its Oδ2. In addition, in molecule B, the positioning of Ser-199(*215*) between the side chains of Asp-89(*102*) and Ser-183(*195*) prevents His-41(*57*) from assuming the active *gauche* conformation, which is characteristic of serine proteases (Figure 5).

These considerations guided the construction of the factor D mutants involving residues T198(*214*) and S199(*215*). The results obtained with the three mutants T198S, S199W, and T198S,S199W (Tables 2 and 3 and Figure 4) suggest that these two residues play a role in substrate binding. This conclusion is based on the more than 3-fold and 2.5-fold increase of the catalytic efficiency of the double mutant against Z-Lys-SBzl and Z-Arg-SBzl, respectively. The results cannot be attributed to a more favorable disposition of the catalytic residues because the reactivity of these mutants toward the dipeptide thioester and their proteolytic activity were similar to those of native factor D. This interpretation is also consistent with the overall low reactivity of the mutants toward small synthetic substrates, as indicated by the more than 1000-fold higher catalytic efficiency of trypsin than the most reative mutant (Table 3). Overall, the effects of these mutations on factor D activity are relatively modest, indicating that other factor D-specific structural features are mainly responsible for its unique catalytic properties.

The functional importance of an additional unique structural feature of the putative substrate binding site of factor D was also explored. A charged Lys residue is found at position 180(*192*) of factor D as compared to a nonpolar Gln or Met present in most other serine proteases (Figure 1). This residue is part of a highly conserved loop that forms the border of the primary specificity binding pocket on the Ser-183(*195*) side. As shown in Figure 5, in molecule B of factor D, the side chain of Lys-180(*192*) extends across the entrance to the primary specificity pocket, probably forming a hydrogen bond with the hydroxyl oxygen of Ser-201(*217*). This disposition raises the possibility of interference of the side chain of Lys-180(*192*) with substrate access to the primary specificity pocket. This structural feature is not observed in molecule A because the ϵ-amino group of the side chain of Lys-180(*192*) is hydrogen-bonded to the carbonyl atoms of residues Glu-45(*60*) and Asp-46(*61*) of the crystallographically related neighboring molecule B in the triclinic unit cell. Obviously, this conformation is the direct result of the crystal packing and probably is not present in native factor D. As shown in Tables 2 and 3 and in Figure 4, mutation of Lys-180(*192*) to Gln resulted in an overall enhanced reactivity, consistent with a negative effect on substrate binding exerted by the disposition of the side chain of Lys-180(*192*) of factor D. Obviously this effect is relatively modest.

The final structural feature of factor D explored could also restrict small synthetic substrate access to the primary specificity pocket of the native enzyme. Similarly to all trypsin-like serine proteases, factor D has as Asp residue at position 177(*189*), which is located at the bottom of the primary specificity pocket (Figure 5). In trypsin, the carboxylate of Asp-177(*189*) forms a salt bridge with the positively charged side chain of the P_1 Arg or Lys residue of the substrate. This salt link is enhanced by the hydrophobic character of the surrounding environment and plays a major role in positioning the scissile bond of the substrate for hydrolysis. In contrast to trypsin, in both molecular forms of factor D the carboxylate of Asp-177(*189*) is not free, but instead it forms a salt bridge with the guanidino group of Arg-202(*218*) (Figure 5). It seems likely that this salt bridge is a major contributor to the low reactivity of factor D toward small synthetic substrates because it restricts access of positively charged side chains to the negative charge of Asp-177(*189*). It thus seems reasonable to suggest that a reorientation of the side chain of Arg-202(*218*) away from Asp-177(*189*) is an important component of the proposed substrate-induced conformational change that leads to efficient proteolysis of C3b-bound factor B. Perhaps this is accomplished through a direct interaction between the side chain of Arg-202(*218*) and negatively charged residues of

the substrate. The results obtained with the R202G,V203Δ mutant are consistent with this hypothesis. Deletion of Val-203(*219*) was coupled to the Arg-202(*218*) to Gly mutation to more closely reproduce this region of trypsin (Figure 1 and 5). Therefore, we cannot draw firm conclusions about the relative contribution of each of the two residues, Arg-202(*218*) and Val-203(*219*), to the observed results. However, the almost complete loss of proteolytic activity of this mutant supports the proposal for a direct interaction between Arg-202(*218*) and C3bB and indicates that this interaction plays a major role in the induction of the proteolytically active conformation of factor D. The markedly reduced proteolytic activity could not be attributed to a conformational change adversely affecting the catalytic triad or the primary specificity pocket of the mutant because its reactivity toward the two single amino acid thioesters was increased (Table 3 and Figure 4). This latter finding is consistent with a negative effect exercised by Arg-202(*218*) on substrate binding. The importance of Arg-202(*218*) and perhaps also Val-203(*219*) in hydrolysis of the natural substrate of factor D is further indicated by the virtual loss of proteolytic activity of the S199W,R202G,V203Δ and the T198S,S199W,-R202G,V203Δ mutants. On the other hand, the failure of these two mutants to show synergistically increased esterolytic activity cannot be readily explained.

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