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REVIEW

Complement factor D, a novel serine protease

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Abstract

Factor D is unique among serine proteases in that it requires neither enzymatic cleavage for expression of proteolytic activity nor inactivation by a serpin for its control. Regulation of factor D activity is instead attained by a novel mechanism that depends on reversible conformational changes for expression and control of catalytic activity. These conformational changes are believed to be induced by the single natural substrate, C3bB, and to result in realignment of the catalytic triad, the specificity pocket, and the nonspecific substrate binding site, all of which have atypical conformations. Mutational studies have defined structural determinants responsible for these unique structural features of factor D and for the resultant low reactivity with synthetic esters.

Keywords: alternative complement pathway; catalytic triad; complement; enzyme kinetics; mutagenesis; serine proteases

Complement is an important effector system of host defense. It consists of multiple, functionally linked proteins that mediate acute inflammatory reactions, clearance of foreign cells and molecules, and killing of susceptible cells. Activation of the complement system is necessary for expression of these activities and proceeds via pathways consisting of successive enzymatic amplification steps (Campbell et al., 1988; Müller-Eberhard, 1988). The key event in complement activation is the cleavage of a single peptide bond on the α-chain of the third component of complement, C3. The reaction is catalyzed by either of two endopeptidases, termed C3-convertases. In the alternative pathway of complement activation, the enzymatic reaction leading to the formation of the C3-convertase is catalyzed by a serine protease, termed factor D. This enzyme exhibits unique functional properties that are ideally suited to its role as the initiating enzyme of an activation cascade. Unlike other mammalian serine proteases in blood, factor D requires neither enzymatic cleavage for expression of proteolytic activity nor inactivation by an inhibitor for its control. Instead, transition of factor D from the catalytically inactive to the active state seems to be effected by fully reversible conformational changes. A description of the structural correlates of these unusual functional features has begun to emerge from recent crystallographic and mutational studies. In this paper, we will review these studies in the context of the functional properties of factor D and of the existing detailed knowledge on the structure and catalytic mechanism of serine proteases.

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Proteolytic activity of factor D

The only known natural substrate of factor D is another complement serine protease, factor B. The single Arg^{233}-Lys^{234} bond of factor B becomes susceptible to factor D-catalyzed hydrolysis only in the context of a Mg^{2+}-dependent complex between factor B and C3b, the major fragment of C3 activation (Müller-Eberhard & Götze, 1972). Cobra factor (CoVF), a C3b analogue present in cobra venom, can efficiently substitute for C3b (Hunsicker et al., 1973). This explains the ability of CoVF to initiate serum C3 cleavage and complement depletion *in vitro* and *in vivo*. Cleavage of C3b-bound factor B by factor D leads to the formation of the C3bBb complex, which is the C3-convertase of the alternative pathway (Fig. 1). Factor D is the only enzyme in blood able to catalyze this reaction and is therefore absolutely required for alternative pathway activation (Lesavre & Müller-Eberhard, 1978).

The concentration of factor D in blood, 1.8 ± 0.4 μg/ml, (Barnum et al., 1984) is the lowest of any complement protein. These low levels make factor D the limiting enzyme in the activation sequence of the alternative pathway. The enzyme becomes nonlimiting only at 9–10 times physiologic serum concentration (Lesavre & Müller-Eberhard, 1978). Thus, factor D is a target of choice for pharmacologic control of com-

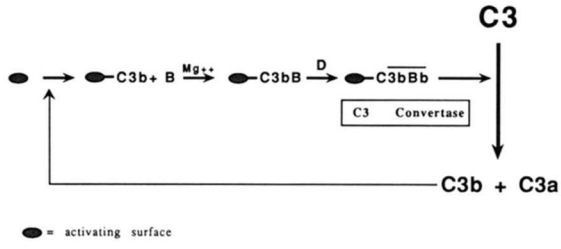

Fig. 1. Formation and amplification of the C3 convertase of the alternative pathway of complement activation. C3b, the major fragment of C3 cleavage is attached to the surface of the activating principle through an ester or amide bond. B, factor B; D, factor D. An overbar indicates proteolytic activity.

plement activation, which could be beneficial in certain human diseases, and efforts to develop specific inhibitors are under way (Kam et al., 1992; Inagi et al., 1994). Studies on patients with renal insufficiency (Volanakis et al., 1985) and *in vivo* microperfusion experiments using rat kidneys (Sanders et al., 1986) have indicated that the low concentration of factor D in blood is maintained by an extremely rapid catabolic rate. Because of its small size, factor D is filtered at high rates through the glomerular membrane and is reabsorbed and catabolized by the proximal tubular epithelial cells. This mechanism results in an estimated fractional catabolic rate of about 60% per hour (Pascual et al., 1988). The high catabolic rate implies a correspondingly high synthetic rate. In that regard, it is interesting to note that adipocytes, formerly thought of as relatively inactive "storage" cells, constitute the main synthetic site for blood factor D (White et al., 1992). This became evident originally for mouse factor D, which for this reason is also referred to as adipsin (Cook et al., 1985; Rosen et al., 1989). Blood monocytes/macrophages (Whaley, 1980; Barnum & Volanakis, 1985) and brain astrocytes (Barnum et al., 1992) can also produce and secrete factor D, which may be important for complement-dependent functions at tissue sites.

Regulation of factor D activity

Mammalian serine proteases are secreted as enzymatically inactive zymogens that require enzyme-catalyzed cleavage of a peptide bond to express activity (Neurath & Walsh, 1976). In pancreatic serine proteases, this process results in the release of a small, 6–15-amino acid residues long, "activation" peptide from the NH_2-terminus of the zymogen. In blood serine proteases, including those participating in complement activation, coagulation, fibrinolysis, and kinin-generation, proteolytic activation of zymogens frequently converts single polypeptides to two-chain molecules. One of the two chains is similar to pancreatic serine proteases, whereas the other subserves other functions usually related to substrate specificity. Activation of zymogens by limited proteolysis obviously provides an important mechanism for regulating catalytic activity (Neurath, 1975).

The primary structure of factor D obtained initially by amino acid sequencing demonstrated that the single-chain, 24-kDa molecule was structurally similar to pancreatic rather than blood serine proteases and that it lacked an activation peptide (Fig. 2) (Johnson et al., 1984; Niemann et al., 1984). A search for a profactor D in blood yielded a trypsin-activatable form of the enzyme (Fearon et al., 1974), which, however, represented only a small percentage of total factor D. It was shown subsequently that only the active form of the enzyme circulates in blood

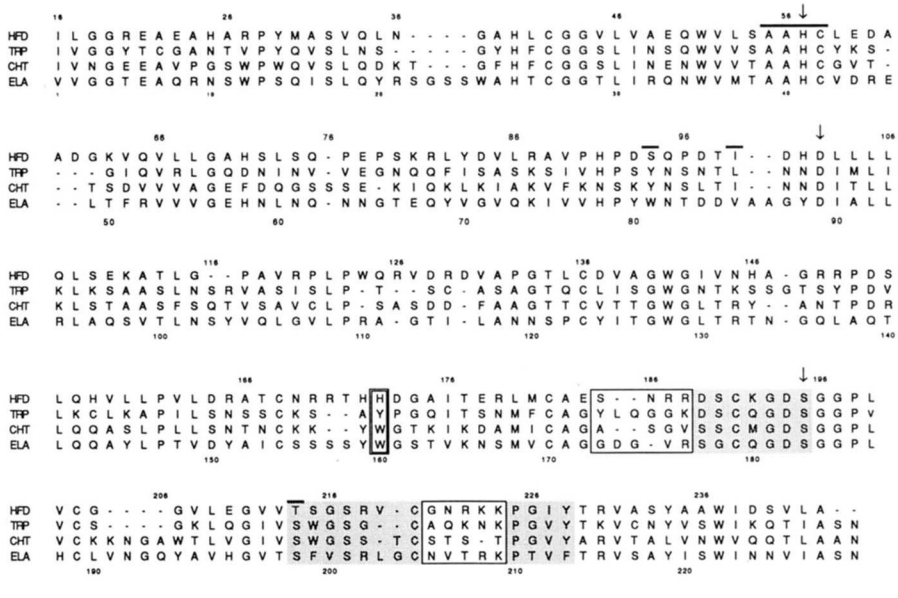

Fig. 2. Alignment of amino acid sequences of factor D and representative pancreatic serine proteases. Arrows indicate catalytic triad residues. Residues participating in shielding the catalytic Asp^{102} from the solvent (Blow et al., 1969) are indicated by overbars. Residues forming the walls of the specificity pocket are shaded and residues forming the surface loops 185–188 (loop 1) and 221–223 (loop 2) are boxed. Residue 172 is boxed by double line. Numbers at the top are for residues of the chymotrypsinogen sequence, and numbers at the bottom are for the factor D residues. HFD, human factor D; TRP, bovine trypsin; CHT, bovine chymotrypsin; ELA, porcine elastase. Sequence data are from Greer (1990), except for the HFD sequence, which is taken from White et al. (1992).

(Lesavre & Müller-Eberhard, 1978). These discordant observations can be reconciled by the recent finding that less than 1% of factor D circulates in proteolytically inactive form (Yamauchi et al., 1994). This "profactor D" has a Gly-Arg dipeptide extension of the NH_2-terminus of the active enzyme, can be activated by high concentrations of trypsin, and it appears to be the product of incompletely processed proprotein. Several observations indicate that the inability to identify and isolate profactor D from blood (Lesavre & Müller-Eberhard, 1978; Davis et al., 1979; Volanakis & Macon, 1987) is not due to an artifact introduced by the purification procedures. First, treatment of plasma with relatively high concentrations of DFP (20 mM) completely inactivates factor D. Second, isolated factor D and factor D in whole serum have the same specific activity (Lasavre & Müller-Eberhard, 1978). Third, administration of CoVF to mice results in complete depletion of circulating C3, indicating the *in vivo* formation of a CoVFBb C3 convertase, which requires the availability of active factor D (Fearon et al., 1974).

Interest in a zymogen form of factor D and its mode of activation was rekindled by the finding that the amino acid sequence deduced from the nucleotide sequence of cDNA clones included not only the mature polypeptide chain and a leader peptide, but also a short additional sequence resembling an activation peptide (White et al., 1992). Expression of this cDNA in Chinese hamster ovary cells yielded active factor D, devoid of the putative activation peptide. However, expression of the same cDNA clone in insect cells by using a baculovirus expression system resulted in secretion of DFP-resistant, proteolytically inactive profactor D consisting of two zymogen forms with respective activation peptides AAPPRGR and APPRGR (Yamauchi et al., 1994). Catalytic amounts of trypsin converted this recombinant profactor D to its enzymatically active form that exhibited specific activity similar to that of native factor D. No auto-activation of profactor D was observed under experimental conditions favorable to its activation by trypsin. In addition, incubation of profactor D with an alternative pathway activator in the presence of serum depleted of factor D did not result in complement activation. Thus, no serum enzyme can interact with the complement activator and convert profactor D to active factor D. Taken together these data support the notion that the activation peptide of profactor D is cleaved-off within the secretory pathway of mammalian cells, similar to many other proproteins (Steiner et al., 1992). Insect cells secrete profactor D apparently because they lack the putative trypsin-like "maturase" or "convertase" (Vernet et al., 1990) responsible for cleaving the activation peptide.

The absence of a circulating profactor D creates the need for an alternative mechanism for regulating factor D activity. An obvious solution to this problem is provided by the extremely restricted substrate specificity of factor D. The single natural substrate of the enzyme, factor B, becomes susceptible to limited proteolytic cleavage by factor D only after it has formed a complex with Mg^{2+} and C3b (Fig. 1). Although it seems likely that formation of the complex induces a conformational change in factor B, this change does not seem necessary for exposing the scissile bond. The Arg^{233}-Lys^{234} bond of native uncomplexed factor B is readily cleaved by trypsin, thus probably exposed to the solvent. It was therefore proposed that "C3b induces factor B to fit into the substrate binding site of factor D," which was envisaged as cryptic (Lesavre & Müller-Eberhard, 1978). An alternative hypothesis proposed that a conformational change of the catalytic center of factor D induced by the C3bB complex is necessary for expression of proteolytic activity (Kam et al., 1987). A corollary to this hypothesis is that, following cleavage of factor B, the active center of factor D reverts to its resting-state inactive conformation. This mechanism would obviate the need not only for a profactor D-activating enzyme in blood, but also for a serpin-type inhibitor, which explains the failure to identify such an inhibitor in blood. The absence of a factor D inhibitor is also indicated by the fact that factor D activity in serum is not diminished by activation of the alternative pathway. Functional support for the substrate-induced active conformation hypothesis has been provided by the seemingly paradoxical observation that, although the proteolytic activity of factor D during complement activation is comparable to that of other complement enzymes, its reactivity with small synthetic thioester substrates and active site inhibitors is three to four orders of magnitude lower than that of typical serine proteases (Kam et al., 1987, 1992).

Esterolytic activity of factor D

A series of peptide thioester substrates that were based on the amino acid sequence preceding the factor B cleavage site were used to map the active site of factor D (Kam et al., 1987). All substrates contained a P_1 Arg residue and various chemical groups and amino acids were used in the P_2, P_3, P_4, and P_5 positions (Schechter & Berger, 1967) to investigate the specificity and reactivity of the enzyme. Of 12 dipeptide thioesters tested, only 3 containing Arg, Val, or Lys in the P_2 position were hydrolyzed at measurable rates. Factor B contains a P_2 Lys at the cleavage site, which is consistent with the finding that dipeptide thioesters with Lys and Arg at P_2 were among the most reactive substrates. That Z-Val-Arg-SBui and Z-Arg-SBzl were also reactive indicates that a hydrophobic group can also fit into the S_2 subsite of the enzyme. More recently, Z-Lys-SBzl was also shown to be reactive with factor D (Kim et al., 1994), indicating that, like trypsin, the P_1 site is reactive with both Arg and Lys residues. Extension of Lys-Arg peptide thioesters to include Gln at the P_3 and P_4 positions, which are also found in the corresponding positions of factor B, resulted in complete loss of reactivity. Neither Bz-Gln-Lys-Arg-SBzl nor Bz-Gln-Gln-Lys-Arg-SBzl were hydrolyzed by factor D at measurable rates. Similarly, tripeptides containing four other amino acid residues at P_3 (Gly, Glu, Lys, Phe) did not react with factor D. Thus, the S_3 subsite of the enzyme is very specific.

In addition to a high degree of specificity, the results of these studies demonstrated that the reactivity of factor D with synthetic esters is extremely low. Overall, factor D was two to three orders of magnitude less reactive (k_{cat}/K_m) than C1s, a serine protease catalyzing a homologous reaction in the classical pathway of complement activation. Compared to trypsin, factor D was as much as 3×10^3 less efficient in hydrolyzing thioester substrates. Factor D exhibited similarly low reactivity toward a series of isocoumarins substituted with basic groups such as guanidino or isothiureidoalkoxy (Kam et al., 1992). These compounds are effective inhibitors for most trypsin-like enzymes examined (Kam et al., 1988). The mechanism of inhibition involves the initial formation of an acyl enzyme. The best inhibitor of factor D was 4-chloro-7-guanidino-3-methoxyisocoumarin, with $K_{obs}/[I]$ value of 250 M^{-1} s^{-1}. By comparison, the inhibition rates of C1s by its best isocoumarin inhibitor were three orders

of magnitude higher, and those obtained with the best inhibitors of trypsin and coagulation enzymes were five orders of magnitude higher (Kam et al., 1988, 1992).

The low reactivity of factor D with synthetic substrates and active site inhibitors supports the proposal of an inactive conformation of native resting-state factor D. Apparently, small peptide thioesters, as opposed to the natural substrate, C3bB, cannot induce the active conformation of the catalytic center. Their reactivity with factor D thus provides a measure of its resting-state inactive conformation. Important structural determinants of the resting-state conformation of factor D have been described recently by X-ray crystallographic and mutational analyses.

Structure of factor D

The three-dimensional structure of several members of the large chymotrypsin family of serine proteases has been described. Examples are provided by the high-resolution crystal structures of chymotrypsin (Birktoft & Blow, 1972), trypsinogen (Fehlhammer et al., 1977), pancreatic elastase (Sawyer et al., 1978), neutrophil elastase (Navia et al., 1989), tonin (Fujinaga & James, 1987), and mast cell protease II (Remington et al., 1988). These studies established that all members of the family have a very similar structure (Greer, 1990). In all cases, the polypeptide chain is folded into two antiparallel β-barrel type domains. Each barrel contains six β-strands that have the same topology in all enzymes.

The crystal structure of factor D was also solved recently by a combination of multiple isomorphous replacement and molecular replacement methods (Narayana et al., 1994). Two molecules, A and B, related by a noncrystallographic twofold axis, are present in the triclinic unit cell. They are very similar to each other as indicated by an average RMS deviation between their main-chain atoms of 1.39 Å. However, the two molecules display distinct orientations of the side chains of the catalytic residues Asp^{102} and His^{57} (chymotrypsinogen numbering has been used throughout this paper). The overall structural fold of both factor D molecules is very similar to that of other serine proteases of the chymotrypsin family. This similarity is illustrated by the superposition of ribbon drawings of factor D and trypsin shown in Figure 3. The only major differences in backbone structure occur in surface loops connecting secondary structural elements. Despite its conserved chymotrypsin fold, factor D displays atypical conformations of key catalytic and substrate-binding residues. Based on current understanding of structural correlates of serine protease functions, these unique structural features would preclude expression of catalytic activity by factor D unless a realignment was induced by conformational changes.

Four features essential for catalysis are present in the structure of all serine proteases: (1) a catalytic triad, (2) an oxyanion-binding hole, (3) a substrate specificity pocket, and (4) a nonspecific binding site that forms a short antiparallel sheet with the substrate (reviewed in Perona & Craik, 1995). These features are arrayed in the same way in all serine proteases of known structure. With the exception of the oxyanion-binding hole, all structural elements of the active site of factor D have atypical conformations.

Catalytic triad

The importance of the three residues, Asp^{102}, His^{57}, and Ser^{195}, which form the "catalytic triad" of serine proteases, was established initially by kinetic analyses, chemical modification experiments (Dixon et al., 1956; Shaw et al., 1965), and structural studies (Blow et al., 1969). The original proposal that these three residues participate in a charge-relay system was not supported by subsequent NMR (Bachovchin & Roberts, 1978) and neutron diffraction studies (Kossiakoff & Spencer, 1981), which demonstrated that no proton transfer occurs from His^{57} to Asp^{102}. However, the need for all three residues for efficient catalysis is well documented. The collective data indicate that substrate hydrolysis is effected by nucleophilic attack of Ser^{195} on the carbonyl carbon of the scissile bond; His^{57} serves as a

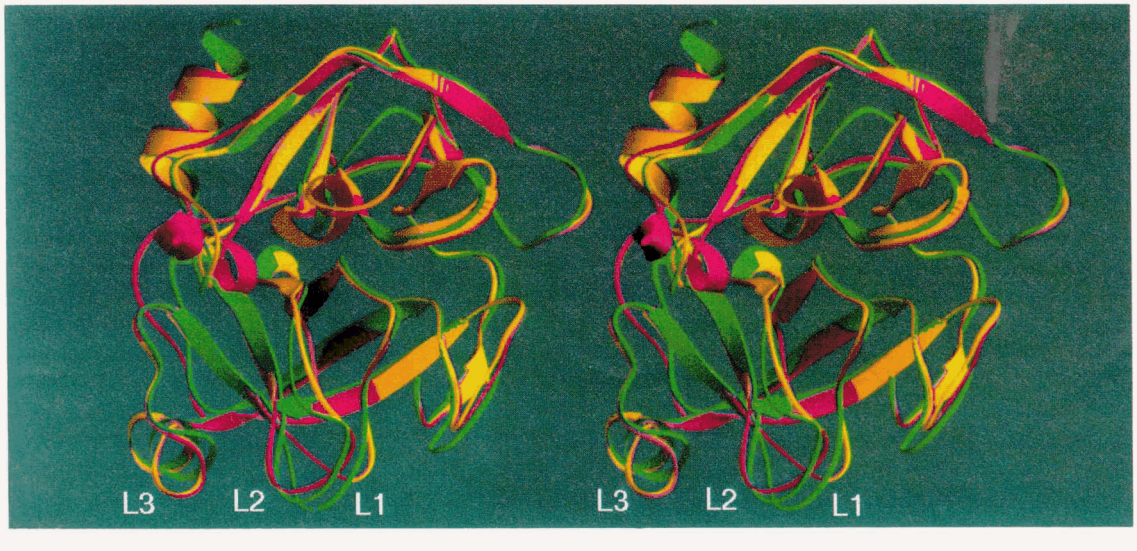

Fig. 3. Stereoscopic view of superposition of the main-chain structures of factor D and trypsin. The models were generated using the ribbons program (Carson & Bugg, 1986). Factor D molecule A, red; molecule B, yellow; trypsin, green. Surface loops 1 (LP1), 2 (LP2), and 3 (LP3), which have been shown to determine substrate specificity in trypsin and chymotrypsin, are identified.

general base catalyst, increasing the nucleophilicity of Ser^{195}; Asp^{102} appears to be involved in maintaining the proper tautomer of His^{57} in the ground state and also in stabilizing the positive charge of His^{57} during the transition state (Craik et al., 1987; Sprang et al., 1987; Carter & Wells, 1988; Warshel et al., 1989). The topology of the catalytic triad residues relative to each other is crucial for their strongly synergistic action. Indeed, the geometric relation of the side chains of the three residues of serine proteases of the chymotrypsin family is invariable.

More impressive is the finding that subtilisin-like serine proteases, which have no overall structural similarity with chymotrypsin-like enzymes, have a similarly arrayed catalytic triad (Wright, 1972). The main feature of the subtilisin structure is a region of five parallel β-strands surrounded by four helices, two on each side of the parallel β-sheet (Wright et al., 1969; Bode et al., 1986). High-resolution refined structures have shown that, in subtilisin and chymotrypsin, the active site aspartates are in different positions with respect to the Ser-His pair and that the H-bonds between Asp and His differ (McPhalen & James, 1988). Nevertheless, in both molecules, the carboxylate of the Asp is almost coplanar with the imidazole ring of the His. It is of further significance that an Asp-His-Ser triad is also employed for catalysis by a third family of proteases, the serine carboxypeptidases, which have a completely different fold of the polypeptide backbone than either chymotrypsin- or subtilisin-like proteases (Liao & Remington, 1990; Liao et al., 1992). Wheat serine carboxypeptidase, the prototype of this third family of serine proteases, has an α plus β fold with 15 helices on either side of a mixed β-sheet consisting of 11 strands, of which the central 6 are parallel. In this enzyme, the carboxylate of the active site aspartate is not coplanar with the imidazole of the His. This finding led to the suggestion (Liao et al., 1992) that the catalytic triad of all serine proteases should actually be regarded as two dyads, a His-Asp dyad and a His-Ser dyad.

The orientation of the side chains of Asp^{102} and His^{57} of factor D are such that neither a chymotrypsin-like or subtilisin-like functional triad nor two carboxypeptidase-like dyads could be supported (Narayana et al., 1994). In molecule A of factor D, the carboxylate of Asp^{102} is pointed away from His^{57} and is freely accessible to the solvent (Fig. 4). However, the geometry of the His^{57}-Ser^{195} dyad is identical to that of chymotrypsin and trypsin (Figs. 4, 5). In molecule B, the imidazolium of His^{57} is oriented away from Ser^{195}, having assumed the energetically favored *trans* conformation (Fig. 4). The space filled in other serine proteases by the imidazolium of His^{57} is occupied by Ser^{215} (Fig. 6). An H-bond between the O^{γ} of Ser^{215} and the O^{δ} of Asp^{102} contributes to the positioning of the side chain of Asp^{102} in a typical serine protease orientation. In other members of the chymotrypsin family, residue 215 is a highly conserved aromatic, usually Trp, which points away from the catalytic triad and is involved in a hydrophobic cluster that also includes Tyr^{172} (Fig. 5). Ser^{195} of molecule B is oriented similarly to other serine proteases. Thus, in molecule B, neither an Asp-His nor a His-Ser catalytic dyad can form.

The finding of two molecular conformations of the catalytic triad of factor D was unexpected. At present it is not clear which one of the two conformations best represents the structure of the enzyme as it exists in the blood. We have recently obtained crystals of factor D in space group $P2_1$ with a single molecule in the asymmetric unit. The structure of this crystal form of factor D has been determined and the model has been refined to an R-factor of 18.8% by using 8.0–2.0-Å resolution data. The catalytic triad of this molecule has a conformation similar to molecule B of the triclinic cell. Similar conformations have also been observed in structures of factor D-inhibitor complexes. These combined data suggest that molecule B is the prevalent form of native factor D. On the other hand, a consideration of the results of mutational analyses of the catalytic triads of subtilisin and trypsin suggests that molecule A also is present in blood. Replacement of the catalytic Ser^{221}, His^{64}, or Asp^{32} of subtilisin with Ala resulted in a decrease of k_{cat} by 2×10^6, 2×10^6, or 3×10^4, respectively (Carter & Wells, 1988). The 100-fold lower values of k_{cat} observed in the Ser and His mutants compared to the Asp mutant were interpreted to be consistent with a more central role of these two residues in catalysis. Mutational analysis of the catalytic triad of trypsin (Craik et al., 1987; Corey & Craik, 1992) yielded very similar results. Substitution of Asn for Asp^{102} resulted in 2×10^3 decrease in k_{cat}, whereas mutants of His^{57} and Ser^{195} displayed a further 10- to 100-fold reduction of k_{cat}. The k_{cat} of factor D for hydrolysis of Z-Arg-SBzl and Z-Lys-SBzl is about 300-fold and 50-fold lower, respectively, than that of trypsin (Table 1; Kam et al., 1987). This result is more consistent with molecule A, which has a typical His-Ser but no Asp-His dyad than with molecule B, in which neither catalytic dyad is conserved. Taken together, the data suggest that probably both molecular forms of factor D exist in blood as alternative conformations.

An understanding of the structural determinants of the unusual geometry of the catalytic triad of factor D has been gained from mutational studies. In their original description of the catalytic importance of the buried Asp^{102} of chymotrypsin, Blow et al. (1969) identified seven residues responsible for shielding Asp^{102} from the solvent. Two of those residues have not been conserved in factor D. A Ser has been substituted for the bulky aromatic Tyr or Trp residue usually present at position 94 of serine proteases and a Thr for the invariant Ser^{214} (Fig. 2). Ser^{214} of chymotrypsin-like proteases is not accessible to the solvent and forms an H-bond with Asp^{102}, thus making an important contribution to its topology (Fig. 5; Warshel et al., 1989). In both molecules of factor D, the hydroxyl group of Thr^{214} is pointed away from Asp^{102}, failing to affect its orientation (Fig. 4). In molecule B, Asp^{102} is apparently stabilized by an H-bond to Ser^{215}, which, as mentioned above, displaces His^{57}, thus disrupting the catalytic triad (Fig. 6).

Mutational studies confirmed the structural contributions of Ser^{94}, Thr^{214}, and Ser^{215} to the unique geometry of the catalytic center of factor D and to its low reactivity with synthetic substrates. 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 Z-Lys-SBzl than recombinant (wt) factor D (Table 1). The increased k_{cat}/K_m could be accounted for by an increase in k_{cat} and could be attributed to a reorientation of the side chains of the catalytic triad residues (Kim et al., 1995a). The high-resolution crystal structure of the S94Y/T214S/S215W factor D demonstrated that the side chains of Asp^{102} and His^{57} have a typical serine protease orientation very similar to that seen in trypsin and chymotrypsin (Fig. 4). A major difference between the latter two proteases and the mutant factor D concerns the positioning of the side chain of Trp^{215}. In both trypsin and chymotrypsin (Fig. 5), Trp^{215} interacts with the highly conserved aromatic (Trp or Tyr) at position 172, which is part of

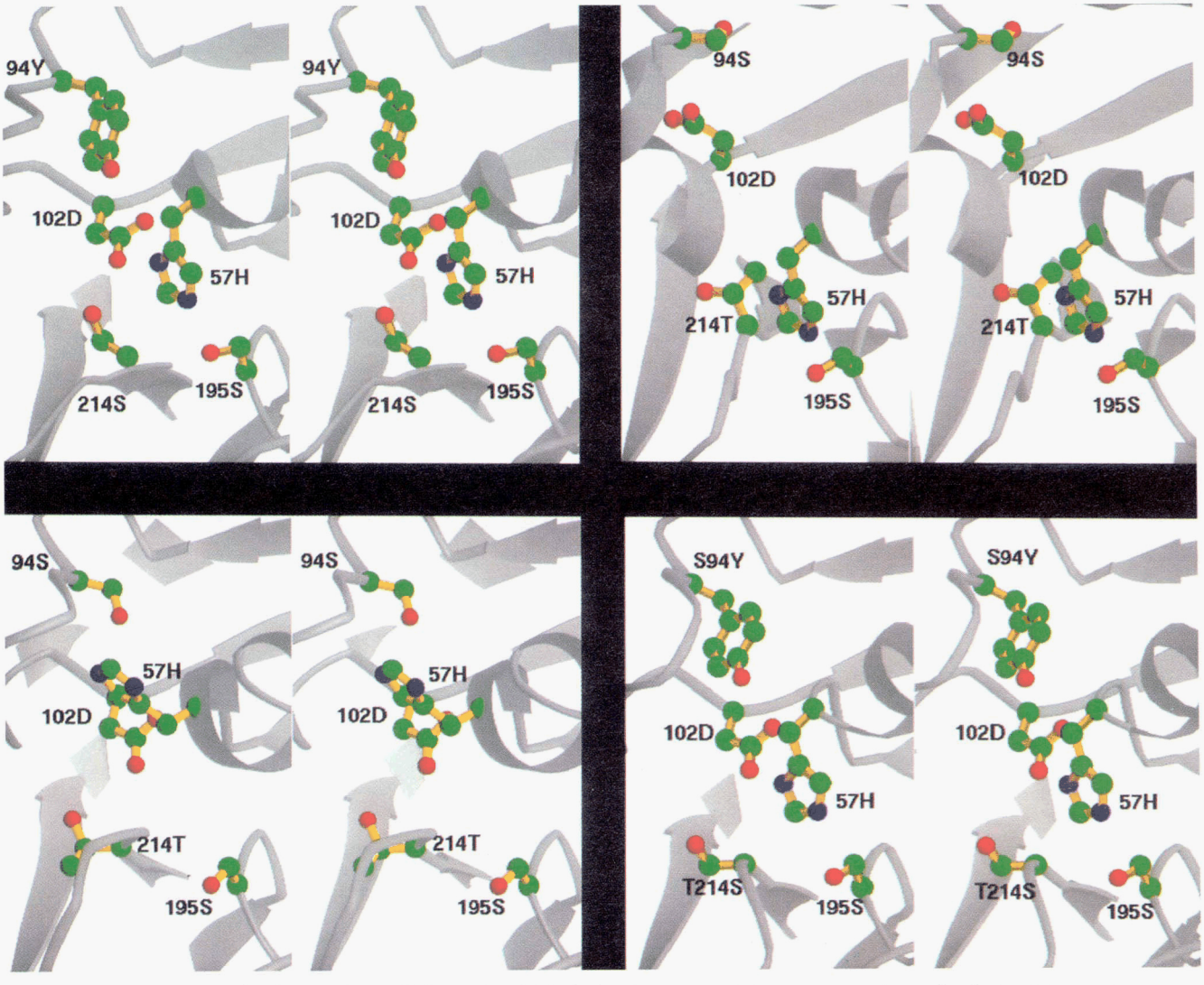

Fig. 4. Stereoscopic view of the catalytic triads of (clockwise from upper left corner) trypsin, molecule A of factor D, S94Y/T214S/S215W factor D, and molecule B of factor D. The unique disposition of the catalytic residues Asp^{102} and His^{57} in factor D can be attributed to the presence of Ser^{94} instead of bulky aromatic Tyr in trypsin and of Thr^{214} of factor D, which fails to form an H-bond with Asp^{102}.

a type I β-turn and has been shown to be a determinant of substrate specificity (Hedstrom et al., 1994; Perona et al., 1995). In the S94Y/T214S/S215W factor D, the loop formed by residues 172–177 is flexible, precluding a hydrophobic interaction between His^{172} and Trp^{215}. Instead, Trp^{215} is oriented in a way favoring hydrophobic interaction with His^{57} (Fig. 7).

All three mutated residues, Ser^{94}, Thr^{214}, and Ser^{215}, apparently contribute to the unusual conformation of the catalytic triad of native factor D and the resulting low k_{cat} for the hydrolysis of small synthetic thioester substrates. However, Ser^{94} seems to be the principal determinant as indicated by the larger effect of the S94Y mutation on k_{cat} for Z-Lys-SBzl than those obtained for the single T214S and S215W mutants (Table 1).

Substrate binding site

Chymotrypsin-like serine proteases have different substrate specificities, yet the three-dimensional structure of their substrate specificity pockets is highly conserved. In γ-chymotrypsin, the walls of the pocket are formed by residues 214–220, 189–195, and 225–228 (Cohen et al., 1981), which exhibit a high degree of similarity to the corresponding residues of trypsin (Fig. 2). The difference in substrate specificity between the two enzymes has been attributed mainly to residue 189 at the bottom of the specificity pocket. Extensive data indicate that the preference of trypsin for Lys and Arg results from the presence of the negatively charged Asp^{189} (Huber & Bode, 1978) and that of chymotrypsin for bulky aromatics from the presence of Ser^{189}. Additional structural elements, particularly the conformation of residue 216, which is a Gly in chymotrypsin and trypsin (Perona et al., 1995), as well as distant surface loops that contribute to the geometry of the specificity pocket (Hedstrom et al., 1992, 1994), play crucial roles in substrate specificity and catalysis.

The presence of an aspartate at the bottom of the specificity pocket defines the entire trypsin-like subfamily of serine proteases, all of which cleave after Arg and/or Lys residues. In tryp-

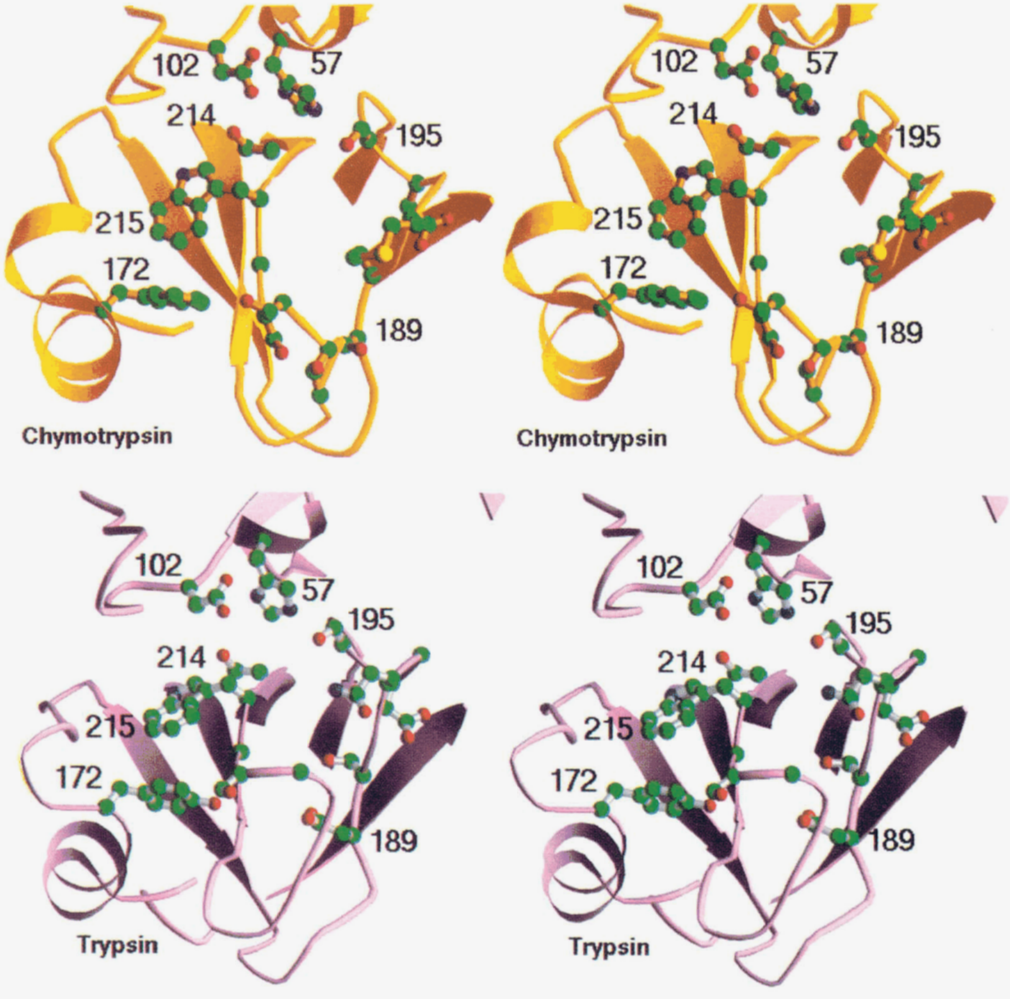

Fig. 5. Stereoscopic views of the specificity pockets of chymotrypsin and trypsin. The models were generated using the ribbons program (Carson & Bugg, 1986). Coordinates for bovine chymotrypsin (4cha) and bovine trypsin (1ptc) were obtained from the Brookhaven Protein Data Bank.

sin, the guanidinium of the P_1 Arg forms an ion pair with the carboxylate of Asp^{189}, whereas the side chain of P_1 Lys interacts with the carboxylate indirectly through a water molecule. Both Arg and Lys side chains also interact with Ser^{190} (Rühlman et al., 1973; Bode et al., 1984). Mutational (Gráf et al., 1987, 1988) and genetic selection (Evnin et al., 1990) analyses, combined with enzyme kinetics and crystallographic studies (Perona et al., 1993, 1994), have confirmed that the presence of a negative charge at the base of the specificity pocket is essential to catalysis by trypsin. Mutants lacking a negative charge are compromised not only in binding affinity (K_s) but also in the rate of acylation (k_2) (Gráf et al., 1988; Perona et al., 1994).

Factor D is categorized as a member of the trypsin subfamily of serine proteases because it cleaves an Arg-Lys bond of its single natural substrate factor B. As expected, an Asp residue is present at the bottom of its specificity pocket. However, unlike other trypsin-like proteases, in both molecules of factor D Asp^{189} forms a salt bridge with Arg^{218} (Fig. 6). It seems likely that this salt link is a major contributor to the low reactivity of factor D with small synthetic substrates, because it restricts access of positively charged P_1 side chains to the negative charge of Asp^{189}. It also seems reasonable to suggest that a reorientation of the side chain of Arg^{218} away from Asp^{189} probably is an important component of the proposed substrate-induced conformational change that leads to efficient proteolysis of C3b-bound factor B.

Mutation of Arg^{218} of factor D to the corresponding trypsin residue Gly combined with deletion of Val^{219}, which is absent from trypsin, resulted in more than 100-fold reduction of proteolytic activity (Table 1). It seems unlikely that the loss of activity was caused by conformational changes of the specificity pocket or of the catalytic triad, because the k_{cat}/K_m of the R218G/V219Δ mutant for hydrolysis of Z-Arg-SBzl and Z-Lys-SBzl was slightly higher than that of native factor D (Table 1; Kim et al., 1994). Therefore, these results were interpreted to indicate a direct interaction between the side chain of Arg^{218} and determinants in the C3bB(Mg^{2+}) complex that could play a role in the induction of the proteolytically active conformation of factor D.

In contrast to the pronounced differences in the conformations of their catalytic triads, molecules A and B of factor D essentially have identical specificity pockets. However, there are

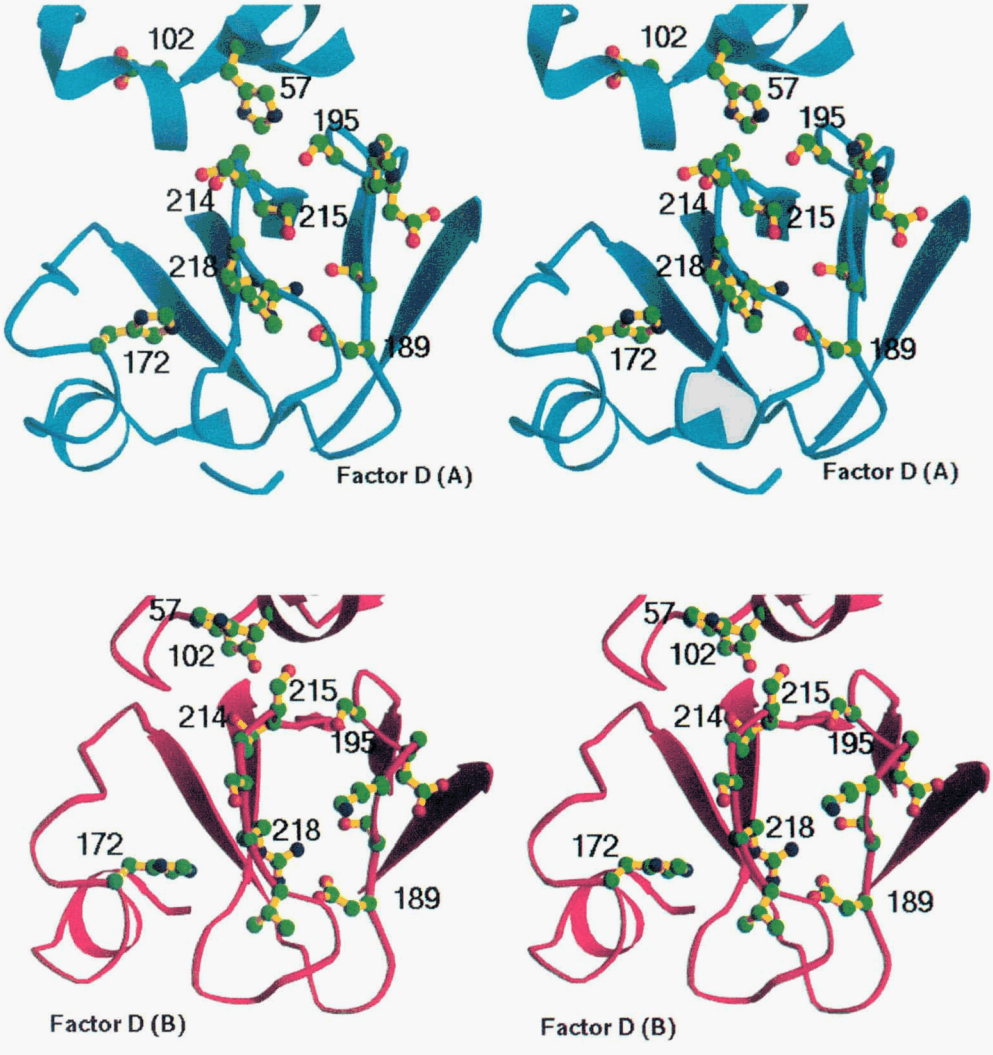

Fig. 6. Stereoscopic views of the specificity pockets of molecules A and B of factor D. The ribbons program (Carson & Bugg, 1986) was used to generate the models. Asp^{189} in both molecules forms a salt link with Arg^{218}. Ser^{215}, which substitutes for the bulky Trp of trypsin and chymotrypsin, is within the pocket in molecule A and forms an H-bond with Arg^{218}. In molecule B, Ser^{215} forms an H-bond with Asp^{102}, forcing His^{57} to assume the *trans* conformation and thus disrupting the catalytic triad.

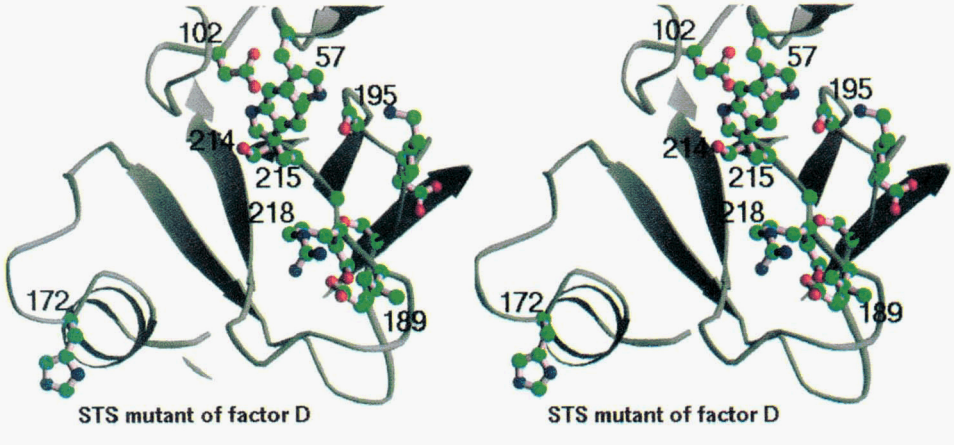

Fig. 7. Stereoscopic view of the specificity pocket of S94Y/T214S/S215W factor D. The model was generated by using the ribbon program (Carson & Bugg, 1986). Asp^{102}-His^{57}-Ser^{195} have a typical serine protease orientation. However, Trp^{215} is oriented away from His^{172}, which is part of a flexible loop. Arg^{218} is pointing away from Asp^{189}, which is H-bonded to Arg^{223}.

Table 1. *Catalytic activity of native and mutant factor D*

Factor D	Hydrolysis of Z-Lys-SBzl			Proteolytic activity[a]
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	(Units/ng)
Native	1.6	4.7	330	24.2
Recombinant (wt)	1.3	3.8	343	26.1
S94Y	7.8	5.2	1,484	140.2
K192Q			391	33.4
T214S	1.8	5.6	326	22.8
S215W	4.5	11.4	394	22.0
S94Y/T214S	12.0	3.3	3,606	136.5
S94Y/S215W	6.3	4.7	1,340	181.9
T214S/S215W	7.1	6.4	1,120	29.8
S94Y/T214S/S215W	21.3	3.0	7,077	245.6
R218G/V219Δ			873	0.2
S215W/R218G/V219Δ			274	0.04
T214S/S215W/R218G/V219Δ			525	0.04
M1[b]	0.3	1.8	161	0.2
M2[c]	0.4	1.6	231	0.06
L1[d]	0.6	5.4	114	2.8
M1-L1	0.8	0.5	1,810	0.07
M2-L1	20.8	0.9	23,961	0.03
Trypsin	74.0	0.065	1.14×10^6	

[a] Measured by a hemolytic assay.
[b] M1:K192Q/T214S/S215W/R218G/V219Δ factor D.
[c] M2:S94Y/M1 factor D.
[d] L1 (loop1), E184G/S185F/185aL/185bE/N186G/R187G/R188K factor D.

two differences between the two molecular forms, which could have an impact on substrate binding. The first involves the orientation of the side chain of Lys^{192}, and the second the positioning of Ser^{215}. The loops containing residues 191–198 of molecules A and B superpose with an average RMS of 0.22 Å in Cα atoms and 0.29 Å in side-chain atoms, excluding the side chain of Lys^{192}. Lys^{192} is the second member of a conserved type II β-turn and, in both molecular forms, its side chain extends into the solvent (Narayana et al., 1994). In molecule A, the ε-amino group of Lys^{192} is H-bonded to the carbonyl atoms of Glu^{60} and Glu^{61} of molecule B in the triclinic unit cell. Obviously, this conformation is the direct result of crystal packing and probably is not present in native factor D. In molecule B, the side chain of Lys^{192} extends across the entrance to the specificity pocket, probably forming an H-bond with the hydroxyl oxygen of Ser^{217}. This orientation raises the possibility of interference with substrate access to the specificity pocket. However, replacement of Lys^{192} with Gln, which is found in trypsin, resulted in only small increases in esterolytic and proteolytic activity (Table 1; Kim et al., 1994).

The difference between molecules A and B in orientation of Ser^{215} is quite striking (Fig. 6). Thr^{214} and Gly^{216} apparently serve as hinges, allowing Ser^{215} to swing in and out of the specificity pocket. In molecule A, Ser^{215} is within the pocket forming an H-bond with Arg^{218}. In molecule B, Ser^{215} has an opposite orientation, occupying the space filled in other serine proteases by His^{57}. Replacement of Thr^{214} or Ser^{215} of factor D with the corresponding trypsin residues Ser or Trp resulted in mutants with esterolytic and proteolytic activities essentially identical to native factor D. The double mutant T214S/S215W had about threefold higher k_{cat}/K_m for the hydrolysis of Z-Lys-SBzl and Z-Arg-SBzl than wt factor D (Table 1; Kim et al., 1994).

Superposition of the specificity pockets of factor D and trypsin (Kim et al., 1995b) reveals that the loops formed by residues 188–195 have very similar backbone conformations, as indicated by an RMS deviation of 0.29 Å between their main chains. Thus, the presence of Lys at position 192 of factor D instead of Gln in trypsin does not affect substantially the conformation of this loop. The oxyanion hole and the topology of the amide nitrogens of Gly^{193} and Ser^{195}, which form H-bonds with the carboxyl oxygen of the substrate in the transition state, are identical between factor D and trypsin. Also, the pocket walls formed by residues 225–228 of factor D and trypsin have very similar conformations. In contrast, the conformation of the third wall of the specificity pocket of factor D, formed by residues 214–220, is considerably different from that of trypsin. As noted above, residues 214 and 215 of factor D are Thr and Ser, respectively, compared to Ser and Trp in trypsin. Residue 218 is Arg in factor D and Gly in trypsin, Val^{219} of factor D is absent from trypsin. Compared to trypsin, the loop 214–219 of factor D is substantially raised up, toward the solvent, with the Arg^{218}-Asp^{189} salt bridge acting as a tether. This positioning of the loop results in considerable narrowing of the specificity pocket.

The atypical conformation of the backbone of loop 214–219 probably is a major determinant of the low reactivity of factor D with synthetic esters. Extensive characterization of mutant trypsins (Hedstrom et al., 1992, 1994) has indicated that the major kinetic determinant of substrate specificity is the rate of acylation, k_2. In turn, a high k_2 depends critically on accurate

positioning of the scissile bond of the substrate relative to the oxyanion hole and the Ser^{195}-His^{57} dyad. Comparative analysis of crystal structures of chymotrypsin, trypsin, and chymotrypsin-like trypsin mutants (Perona et al., 1995) indicated that the main-chain conformation of Gly^{216} is a crucial structural determinant for the correct orientation of the scissile bond. In all serine proteases, residue 216 forms two antiparallel β-strand H-bonds with the P_3 residue of the substrate. Both chymotrypsin and trypsin have a Gly at position 216, but the H-bonds formed between this residue and P_3 differ between the two enzymes and are believed to act as specificity determinants. In factor D, a Gly is also present at position 216, but its backbone conformation is clearly different from that of trypsin. This fact may partially explain the low reactivity of factor D with thioesters and, in particular, its inability to cleave tri- and tetra-peptide thioesters (Kam et al., 1987).

Efforts to increase the esterolytic activity of resting-state factor D by replacing residues lining the specificity pocket with those present in trypsin were met with little or no success (Table 1). Indeed, K192Q/T214S/S215W/R218G/V219Δ (M1) factor D, which has a specificity pocket essentially identical to that of trypsin, has slightly reduced k_{cat}/K_m for hydrolysis of Z-Lys-SBzl than native factor D. Combining these mutations with the S94Y mutation (M2) failed to improve reactivity. Yet, as mentioned previously, the partial S94Y/T214S/S215W mutant has a 20-fold higher k_{cat}/K_m than native factor D. These results suggested strongly that structural elements outside the pocket determine substrate specificity and reactivity. A similar conclusion was arrived at in studies aimed at converting the specificity of trypsin to that of chymotrypsin (Gráf et al., 1988). It was shown subsequently that, in addition to binding pocket mutations, conversion of specificity required mutational replacement of two surface loops (Hedstrom et al., 1992) and of residue 172 (Hedstrom et al., 1994). The two loops formed by residues 185–188 (loop 1) and 221–224 (loop 2) (Fig. 2) act synergistically with the specificity pocket to determine substrate specificity without contacting the substrate directly. Crystallographic studies (Perona et al., 1995) demonstrated that loop 2 specifies the main-chain conformation of Gly^{216}, which, as mentioned above, is an important specificity determinant. These studies also showed that residue 172, which is Tyr in trypsin and Trp in chymotrypsin, defines the geometry of the base of the specificity pocket. This residue also constitutes a major substrate specificity determinant.

In factor D, residue 172 is His and is a member of a rigid type I β-turn (Fig. 6). Its contribution to substrate specificity and catalysis has not been investigated. Loop 2 of factor D has a sequence similar to that of trypsin and the two loops superpose quite well (Figs. 2, 3; Kim et al., 1995b). However, loop 1 of factor D is shorter by two residues than that of trypsin and has a distinct amino acid sequence. Similarly, the backbone conformation of loop 1 of factor D is quite different from that of trypsin (Figs. 2, 3). It has been suggested (Kim et al., 1995b) that the positioning of loop 1 may contribute to the upward extension of loop 214–220 of factor D, which results in narrowing of the specificity pocket. Mutational replacement of loop 1 of factor D with that of trypsin resulted in a factor D mutant (E184G/S185F/185aL/185bE/N186G/R187G/R188K;L1), which had slightly lower k_{cat}/K_m than native factor D for the hydrolysis of Z-Lys-SBzl. This was due mainly to a lower k_{cat}. The proteolytic activity of this mutant, measured by a hemolytic assay, also was reduced (Table 1). We considered the latter result to be consistent with a role of loop 1 in determining the tertiary structure of the substrate specificity pocket (Kim et al., 1995b). Combining the loop 1 mutant with the specificity pocket M1 and M2 mutants (Table 1) resulted in markedly increased reactivities, with k_{cat}/K_m of one and two orders of magnitude higher, respectively, than native factor D. Interestingly, the increased reactivity of the M1-L1 mutant was mainly caused by a 9-fold decrease of K_m, whereas that of the M2-L1 mutant resulted from the combined effect of an 18-fold increase in k_{cat} and a 5-fold decrease in K_m. Compared to trypsin, the M2-L1 factor D was only about 45-fold less reactive with the thioester substrate. In contrast to their enhanced esterolytic activity, the proteolytic activity of these mutants was two to three orders of magnitude lower than that of native factor D. This can probably be attributed to the combination of the $Arg^{218} \rightarrow Gly$ mutation and the deletion of Val^{219}. These two mutations, whether combined with additional ones or not, always cause a great reduction of proteolytic activity. The impressive dissociation between proteolytic and esterolytic activity of these mutants supports the proposal that the latter reflects the resting-state, "uninduced" conformation of factor D.

Conclusions

Recent crystallographic studies have provided strong support for the proposed novel mechanism of regulation of factor D activity. Several structural features apparently account for the low reactivity of native factor D with synthetic substrates. They include the atypical orientation of the side chains of the catalytic residues Asp^{102}/His^{57}, a salt bridge between Arg^{218} and Asp^{189}, the specificity residue of the substrate-binding pocket, the unusual conformation of the loop 214–220, which obstructs the pocket, and the atypical main-chain conformation of Gly^{216}. Mutational studies have identified structural elements responsible for these unique active-site conformations and for the low reactivity of resting-state factor D. They include residues lining the specificity pocket (Lys^{192}, Thr^{214}, Ser^{215}, Arg^{218}, and Val^{219}), residues forming the surface loop 184–188 (Glu^{184}, Ser^{185}, Asn^{186}, Arg^{187}, Arg^{188}), and Ser^{94}. Results of esterolytic assays indicate that these residues act synergistically. Trypsin-like replacement of these residues results in substantial increase of k_{cat}/K_m. It seems reasonable to speculate that the structural changes caused by these mutations are, at least in part, similar to the putative substrate-induced conformational changes that seem necessary for expression of proteolytic activity during complement activation.

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