Mutational Analysis of the Primary Substrate Specificity Pocket of Complement Factor B

ASP226 IS A MAJOR STRUCTURAL DETERMINANT FOR P1-ARG BINDING*

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Factor B is a serine protease, which despite its trypsin-like specificity has Asn instead of the typical Asp at the bottom of the S₁ pocket (position 189, chymotrypsin numbering). Asp residues are present at positions 187 and 226 and either one could conceivably provide the negative charge for binding the P₁-Arg of the substrate. Determination of the crystal structure of the factor B serine protease domain has revealed that the side chain of Asp226 is within the S₁ pocket, whereas Asp187 is located outside the pocket. To investigate the possible role of these atypical structural features in substrate binding and catalysis, we constructed a panel of mutants of these residues. Replacement of Asp187 caused moderate (50–60%) decrease in hemolytic activity, compared with wild type factor B, whereas replacement of Asn189 resulted in more profound reductions (71–95%). Substitutions at these two positions did not significantly affect assembly of the alternative pathway C3 convertase. In contrast, elimination of the negative charge from Asp226 completely abrogated hemolytic activity and also affected formation of the C3 convertase. Kinetic analyses of the hydrolysis of a P₁-Arg containing thioester by selected mutants confirmed that residue Asp226 is a primary structural determinant for P₁-Arg binding and catalysis.

Complement is a major effector system of host defense. Activation of complement leads to the generation of protein fragments and protein-protein complexes that mediate acute inflammatory responses, phagocytosis and killing of pathogens, and regulation of adaptive immune responses. Activation-associated production of biologically active protein fragments is catalyzed by a group of eight atypical complement serine proteases (SPs) of the chymotrypsin superfamily (1). Understanding the structural basis for the highly restricted proteolytic activity of these SPs is an important first step toward pharmacologic control of complement activation (2).

Members of the chymotrypsin family have very similar three-dimensional structures but distinct substrate specificities. To a great extent specificity is determined by the side chains of the amino acid residues that line up the primary substrate specificity pocket (S₁ site). The pocket has three walls formed by residues 189–195, 214–220, and 225–228 (chymotrypsin numbering has been used for all SPs or SP domains throughout this paper) (3). The presence at the bottom of the pocket of Asp189 endows trypsin with preference for positively charged Arg and Lys residues (4, 5), whereas in chymotrypsin the specificity for bulky aromatics is largely determined by Ser189 (6). Residues at position 216 and 226 also contribute to substrate specificity (7). All complement SPs exhibit trypsin-like specificity for positively charged Arg residues and all have an Asp at position 189, except for factor B and C2 (Fig. 1).

Factor B and C2 are structurally similar modular proteins that play a central role in complement activation by providing the catalytic subunits of two key enzymes, namely the C3/C5 convertases of the alternative and the classical pathway, respectively. Complement convertases cleave the same single peptide bonds in C3 and C5. In addition to having Asn and Ser, respectively, instead of Asp at position 189, factor B and C2 also lack the highly conserved free N-terminal sequence of SPs. In typical SPs, the N-terminal sequence constitutes an essential structural element largely responsible for the transition from zymogen to active enzyme (8). Full expression of the proteolytic activities of factor B and C2 only occurs in the context of the complexes, C3bBb(C3b) and C4b2a(C3b), respectively (9). The SP domain resides in the C-terminal half of Bb or C2a and is preceded by a von Willebrand factor type A module (VWFA) which is noncovalently associated with C3b or C4b, respectively, in a Mg²⁺-dependent manner. These atypical structural features of factor B and C2 indicate a novel activation mechanism and probably also a distinct substrate binding arrangement at the primary specificity pocket.

In addition to their natural protein substrates C3 and C5, factor B and C2 and their fragments Bb and C2a hydrolyze a small number of C3- and C5-like synthetic substrates (11–14). Overall, C3-like substrates are considerably more reactive than C5-like substrates. However, even toward their best substrates, the $k_{\text{cat}}/K_m$ values of factor B, Bb, C2, and C2a are much lower than their $k_{\text{cat}}/K_m$ values towards C3b. The $K_m$ values of factor B, Bb, C2, and C2a for C3b are in the µM range, whereas the $K_m$ values of factor B, Bb, C2, and C2a for C3a are in the mM range (15). This preferential activity toward C5-like substrates compared with C3-like substrates is typical for the other complement C3/C5 convertases of the alternative and the classical pathway.
about 3 orders of magnitude lower than the 7.8 $\times$ 10$^{8}$ s$^{-1}$ M$^{-1}$ value measured under the same conditions for the hydrolysis of the most reactive thioester by trypsin (14). By comparison, the catalytic efficiency $(k_{cat}/K_{m})$ of C3bBb for C3 cleavage was reported to be 3.1 $\times$ 10$^{8}$ s$^{-1}$ M$^{-1}$ (10). No natural serine protease inhibitor has been found for factor B or C2 and regulation of the conformation change(s) associated with zymogen activation and extremely restricted substrate specificity as well as the conformational change(s) associated with zymogen activation are not understood. Determination of the structure of the factor B serine protease domain (B-SP) at 2.1-Å resolution has revealed the expected chymotrypsin fold but also unique features of surface loops and of the oxyanion hole. The backbone conformation of the S$_1$ pocket is similar to that of trypsin, but there are substitutions of functionally important residues. In this study we used site-directed mutagenesis to analyze possible effects of the factor B-specific residues on the assembly and activity of the C3 convertase. The data indicate that Asp$^{226}$ is a primary structural determinant of P$_1$-Arg binding and that the native conformation of Asp$^{226}$ and Asn$^{189}$ are important determinants for C3 cleavage.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutant Factor B cDNA**—The factor B cDNA clone BH4-1 (15) in the expression vectors pReCMV or pcDNA3 (Invitrogen, Carlsbad, CA) was used as wild type (wt) template in site-directed mutagenesis. Factor B mutant cDNA constructs were obtained by the method of Zollar and Smith (16) as modified by Kunkel (17). Alternatively, the QuikChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's protocol. All cDNA constructs of mutant factor B were verified by restriction mapping and dideoxynucleotide sequencing (18) of the region around the mutation. Oligonucleotides were synthesized by the phosphoramidite method (19), using a DNA/RNA synthesizer (Model 394 Applied Biosystems, Foster City, CA).

**Expression of wt and Mutant Factor B cDNA**—Transient transfection of COS cells with 30–40 µg of cDNA was performed by electroporation as described (20). Cell culture supernatant containing secreted factor B proteins was harvested 72–90 h after transfection. Cell debris was removed by centrifugation and the supernatant was stored frozen at −80 °C in small aliquots. The concentration of recombinant factor B in the medium was measured by enzyme-linked immunosorbent assay (15), using a rabbit anti-human Bb IgG (50 µg/ml) as capturing antibody and the mouse anti-Ba monoclonal antibody (mAb) HA4-1D5 (1.5 µg/ml) as reporter. The assay was developed with 1:1000 dilution of affinity-purified goat anti-mouse IgG1 alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL) and Sigma substrate Sigma 104. Color development was measured at 405 nm. The concentration of factor B was calculated from a standard curve constructed using human serum of known factor B concentration. The sensitivity of the assay was approximately 1–2 ng/ml and the concentration of specific protein in the culture medium ranged from 0.3 to 2 µg/ml.

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RESULTS

To understand the structural implications of the unique factor B residues in and around the primary specificity pocket, the serine protease domain (B-SP) was expressed using a baculovirus system and its crystal structure determined at 2.1-Å resolution by multiple isomorphous and molecular replacement methods. As expected, B-SP was found to display a chymotrypsin-like, two β-barrel structural fold. In the active center, the catalytic triad residues, Asp<sup>189</sup>, His<sup>57</sup>, and Ser<sup>195</sup>, have typical serine protease configurations (Fig. 2). However, the oxyanion hole displays a zymogen-like conformation due to the inward orientation of the carbonyl oxygen atom of Arg<sup>192</sup>, the backbone of which together with those of Cys<sup>191</sup>, Gly<sup>193</sup>, and Asp<sup>194</sup> form a single-turn 3<sub>10</sub> helix. The three walls of the primary specificity pocket are formed by residues 189–195, 214–220, and 225–228. The backbones of these residues, except for the single-turn helix, can be superposed on those of the corresponding residues of trypsin. Asp<sup>189</sup> is located at the bottom of the pocket, replacing the highly conserved Asp of other SPs with trypsin-like substrate specificity. However, the side chain of Asp<sup>226</sup>, which replaces Gly<sup>226</sup> of trypsin, extends toward the bottom of the pocket which suggests that it may be directly involved in binding the P<sub>1</sub>-Arg of the substrate substituting for Asp<sup>189</sup> of other trypsin-like SPs. An Asp residue also replaces a conserved Gly of other SPs at position 187. Asp<sup>187</sup> of factor B is located directly beneath the pocket and forms a salt bridge with Lys<sup>162</sup>. To investigate the possible participation of the three residues, Asp<sup>187</sup>, Asp<sup>189</sup>, and Asp<sup>226</sup>, in substrate binding and catalysis, factor B mutants at these positions were constructed and assayed. In addition, the functional role of Pro<sup>188</sup>, not found at this position in other SPs, was also assessed. In most cases, two independent clones for each mutant were expressed and analyzed to avoid artificial results. In all cases, results of functional analysis of the two clones of each mutant were consistent. This suggested that functional differences from the wt resulted from the amino acid substitution at the mutation sites.

Reactivity of Factor B Mutants with Module-specific mAbs—To probe for possible effects of the mutations on the overall structure of the molecule, we tested the reactivity of the mutants with a panel of module-specific mAbs. The anti-Bb mAb HA4–15 (22) has been shown to recognize an epitope on the SP domain (data not shown). MAbs FD3–20 (anti-CCP1–3) and HA4–1D5 (anti-CCP2) bind to distinct epitopes on the Ba segment (29), while 6B3.3 (γ1,κ) recognizes an epitope on the VWFA module at or near the C3b-binding site (data not shown). We did not observe substantial differences in the reactivity of the mutants with the four mAbs (data not shown), suggesting that all epitopes tested are retained in their native conformation.

Formation of the CoVFB and CoVFBb Complexes—Expression of proteolytic activity by the factor B SP domain requires binding of factor B to C3b and its proteolytic cleavage by factor D. Introducing mutations in the SP domain could alter C3b binding and/or susceptibility to factor D cleavage, although these functions have been assigned to distal parts of the molecule, namely, the CCP and the VWFA modules (1). We examined the ability of factor B mutants to form the CoVFB and CoVFBb complexes. Choice of CoVFB over C3b was dictated by the much longer half-life of the complexes, which facilitates detection. All mutants showed dose-dependent binding to CoV in the absence (data not shown) and presence (Fig. 3) of factor D. Enhancement of binding to CoVFB was observed in the presence of factor D for all mutants. Factor B carrying single mutations at positions 187 or 189 had essentially the same binding activity as wt factor B, except for the D187Y mutant, which only formed about half as much CoVFBb as wt factor B. In the D226 panel of mutants, surprisingly only D226N had wt binding activity. The same substitution combined with N189D mutations at positions 187 or 189 had essentially the same binding activity as wt factor B, except for the D187Y mutant, which only formed about half as much CoVFBb as wt factor B. In the D226 panel of mutants, surprisingly only D226N had wt binding activity. The same substitution combined with N189D resulted in 50% reduction of binding to CoV compared with either the D226N or N189D mutant. The trypsin-like mutation D226G alone or in combination with the N189D mutation...
caused 60 and 87% reduction, respectively, in CoVFBb complex formation. Similar reductions in CoVF binding ability of the mutants was also observed without factor D cleavage (data not shown). The results suggested that, with the exception of the D226N mutation, substitutions at position 226 affect initial binding of factor B to CoVF thus sensitivity to factor D proteolysis, since binding is a prerequisite for factor B cleavage. In a more direct factor B cleavage assay, conversion of biosynthetically labeled factor B to Bb by factor D in the presence of CoVF was analyzed by SDS-PAGE and autoradiography (Fig. 4). The results correlated well with the binding data. Mutant D226N was as sensitive to factor D cleavage as wt factor B. Mutants D226N/N189D, D226G, and D226G/N189D were less susceptible to factor D with conversion to Bb estimated at 53, 27, and 16%, respectively, of that of wt factor B at the high concentration of factor D. The combined results suggest that although the overall structural integrity of the mutants was preserved, as indicated by equivalent reactivity with the module-specific mAbs, amino acid substitutions in the SP domain apparently affected CoVF/C3b binding, which is mediated by sites on the other two domains of the molecule.

Hemolytic Activity of Factor B Mutants—The effects of the mutations on the ability of factor B to cleave/activate C3 and C5 were assessed by a hemolytic assay. The hemolytic activity of the mutants relative to that of wt factor B is illustrated in Fig. 5. Elimination of the negative charge of Asp187 in mutants D187A, D187N, and D187S resulted in 50–60% loss of hemolytic activity. Substitution of Tyr at the same position caused a more pronounced decrease in hemolytic activity, approximately 80%. The data suggest that the bulky hydrophobic side chain of Tyr is not favored and that full expression of factor B hemolytic activity requires the salt-bridging conformation of Asp187. Ala mutation at position 188 in the mutant P188A did not have significant effect on the hemolytic activity. As revealed in the crystal structure, Asn189 and the side chain of Asp226 are located at the bottom of the primary specificity pocket and appear to be accessible to the P1-Arg of the substrate (Fig. 2). Replacement of Asn189 with charged residues, either Asp or Lys, reduced hemolytic activity by 95%, while the Ala mutant retained approximately 30% of wt activity. Although eliminating the negative charge from Asp226 in the D226N mutant did not affect the assembly of the CoVFBb complex (Fig. 3), it completely abrogated the C3/C5 convertase activity. Replacement of the same residue with Gly present in trypsin also resulted in complete loss of hemolytic activity. Again the loss of hemolytic activity was out of proportion to the only moderately reduced ability to form the CoVFBb complex (Fig. 3). Attempts to construct a trypsin-like pocket by reassigning the negative charge to position 189 in the double mutants D226N/N189D and D226G/N189D failed to restore factor B hemolytic activity, despite the residual CoVF binding activity (Figs. 3 and 5). The hemolytic data strongly indicate that Asp226 plays a critical and highly specialized role in the expression of C3/C5 convertase activity by factor B. Residue Asn189 and Asp187 are also of importance for expression of factor B-dependent proteolytic activity. In contrast, the Pro residue at position 188 has no apparent functional role and likely serves as spacer between structurally crucial residues.

C3 Cleavage Assay—Decrease of the factor B hemolytic activity could reflect a defect of C3 and/or C5 cleavage. The effects
of the mutations on C3 proteolytic activity were assessed by a direct cleavage assay. Wt factor B and selected mutants were permanently expressed in CHO cells and purified. Fluid-phase C3 convertases were formed with CoVF in the presence of factor D (1.5 μg/ml) at 37 °C for 2 h. CoVF-bound Bb fragments were detected by using rabbit anti-human Bb IgG and goat anti-rabbit IgG as detailed under “Experimental Procedures.” Symbols are: A, wt B; ●, D187A; ▲, D187N; ●, D187Y; B, ■, wt B; ▲, N189A; ●, N189D; ▲, N189K; C, ■, wt B; ●, D226N; ○, D226N/N189D; ▲, D226G; ▼, D226G/C; ▲, D226G/N189D.

FIG. 3. Assembly of solid-phase CoVFBb complex by wt and mutant factor B. Microtiter plates were coated with CoVF (10 μg/ml). Serial dilutions of wt and mutant factor B in culture supernatants of transfected COS cells were added and incubated with factor D (1.5 μg/ml) at 37 °C for 2 h. CoVF-bound Bb fragments were detected by using rabbit anti-human Bb IgG and goat anti-rabbit IgG as detailed under “Experimental Procedures.” Symbols are: A, ■, wt B; ●, D187A; ▲, D187N; ●, D187Y; B, ■, wt B; ▲, N189A; ●, N189D; ▲, N189K; C, ■, wt B; ●, D226N; ○, D226N/N189D; ▲, D226G; ▼, D226G/C; ▲, D226G/N189D.

DISCUSSION

Determination of the structure of the SP domain of factor B revealed a number of novel insertions and deletions compared with typical SPs and also certain unique structural features of the catalytic apparatus, especially in the primary specificity pocket (data not shown). In the present study, mutational analysis of factor B residues in and around the primary specificity pocket was performed to investigate structural correlates of substrate recognition at the S1 site. The results are discussed in light of the large amount of available information on SP specificity.

Our results clearly demonstrate that Asp226 of factor B is a critical structural determinant for substrate binding and catalysis, substituting for Asp189 in other SPs with trypsin-like specificity. Functional analysis of the D226N mutant revealed the most clear-cut results. The observed loss of esterolytic and proteolytic activity of this mutant could be attributed solely to a catalytic defect resulting from inappropriate engagement of the P1-Arg in the S1 site, while other functional sites necessary for the proteolytic activation and substrate binding appeared to be well preserved. A sharp 50-fold decrease in catalytic rate (kcat) indicates that a negative charge at the bottom of the
primary pocket is essential for efficient catalysis, but not for overall substrate binding affinity, because the $K_m$ is not altered by the Asn substitution (Fig. 7). Apparently, hydrogen bond formation of the P1-P3 residues to the nonspecific substrate-binding site, Ser-Try-Gly214–216, and hydrophobic anchoring of the P2 and P3 side chains to $S_2$ and $S_3$ pockets, respectively, provide sufficient binding force. Also it seems likely that Asn226 provides additional binding energy, probably by hydrogen bonding with P1-Arg. However, positioning of the scissile bond relative to Ser 195 and the oxyanion hole through the putative hydrogen bonds may differ from that effected by the direct ionic contact made by Asp226 in wt factor B. Replacing Asp 226 with Asn affected equally esterolytic and C3 proteolytic activity, although D226N factor B could form a CoVFBb complex. In a recent report Hourcade et al. (30) also found that substitution of various residues (Asn, Ala, Ser, and Tyr) for Asp 226 caused severe reduction in proteolytic activity despite normally assembled C3bBb complex. It is of special interest that the conservative substitution of Glu for Asp226 also abrogated C3 catalytic activity. This observation suggests that accurate positioning of the carbonyl group of P1-Arg of C3 relative to the nucleophilic Ser195 $O^\gamma$ and oxyanion hole can only be achieved by the native residue Asp226. A corresponding trypsin mutant, D189E, displayed 2–3 orders of magnitude decrease in catalytic efficiency ($k_{cat}/K_m$), associated with a 40-fold shift in the preference from Arg to Lys substrates relative to wt trypsin (31). Apparently, the additional methylene group distancing the carboxylate of trypsin D189E from the peptide backbone within the narrow S1 pocket impeded the proper positioning of the side chain of Arg, which is longer and larger than that of Lys. The loss of C3 catalytic activity by D226E factor B (30) can probably be attributed to a similar spatial effect.

Another structural characteristic of the $S_1$ pocket of factor B is a hydrogen bonding network formed by the carboxyl oxygens of Asp226 and pocket residues Asn189, Thr190, and Arg225 (Fig. 7). This effectively reduces ionic bonding potential available for making contacts with P1-Arg of the substrate. On one hand, this distinct feature could possibly explain the overall low esterolytic activity of factor B, Bb (12–14), and B-SP (Fig. 7). On the other hand, it implies the need for additional bonding between P1-Arg and other pocket residues. The side chain of Asn189 faces the carboxyl of Asp226 from the opposite wall and occupies a central position at the bottom of the specificity pocket. Although the position of the Asn189 side chain is about 0.5–1.0 Å lower than that of Asp226, it appears accessible to the substrate. Our results indicate a supporting role for Asn189 in substrate recognition and catalysis. Substitution of Ala, Asp, or Lys at this position caused substantial reduction or abrogation of hemolytic activity, which paralleled a similar reduction in C3.
proteolytic activity (Figs. 5 and 6). The Ala substitution caused a decline in synthetic substrate binding affinity ($K_m$) and catalytic efficiency ($k_{cat}/K_m$), which strongly indicates participation of Asn$^{189}$ in substrate recognition. The amine group of the Asn$^{189}$ side chain may mediate P$_1$-Arg binding through a hydrogen bond. Absence of this potential binding force may compromise accurate register of P$_1$-Arg of C3 for catalysis. Substitution of a charged residue, Asp or Lys for Asn$^{189}$ in N189D and N189K, respectively, abrogates C3 proteolytic activity of the C3- or CoVF-bound Bb. Interestingly, the N189D mutant retains substantial esterolytic activity toward the synthetic substrate. These results suggest that the reconstructed S$_1$ pocket, with free carboxyls at positions 226 and 189, despite its altered geometry could register to the His$^{57}$-Ser$^{195}$ dyad, the Arg bond of the synthetic substrate but not that of C3. The free leading or leaving group of the synthetic substrate may account for the observed binding flexibility.

C2 and factor B have identical proteolytic specificity for single Arg peptide bonds of C3 and C5 so that their substrate-binding sites can be presumed to be very similar in geometry and chemical nature. Thus, it is not surprising that C2 has Asp and Ser at positions 226 and 189, respectively (Fig. 1). Besides Arg and C2, an acidic residue is also present at position 226 in a few additional members of the chymotrypsin family, namely fiddler crab collagenase (cCOLL) (32), human cathepsin G (CATG) (33), protease 3 (hPRO3) (34), and neutrophil elastase (hnELA) (35). In contrast to C2 and factor B these serine proteases display relatively broad substrate specificity. cCOLL and CATG recognize not only basic but also large hydrophobic side chains (32, 36). The Arg/Lys substrate preference is mainly attributed to the presence of Asp$^{226}$/Glu$^{189}$ in cCOLL and of Glu$^{226}$/Ala$^{189}$ in CATG within the S$_1$ pocket. The large and flexible S$_1$ pocket in cCOLL allows this enzyme to adjust to different shapes of the P$_1$ side chain. Removal of the negative charge from the cCOLL S$_1$ pocket in the D226G mutant resulted in a significant decrease of catalytic efficiency toward Arg/Lys substrates (37). Similarly to Asp$^{226}$ in factor B and cCOLL, the corresponding Glu$^{226}$ in human CATG has only one carboxyl oxygen available for substrate binding (33). This may be responsible for the relatively slow catalysis of substrates with P$_1$-Lys or Arg. However, the presence of a negatively charged residue at position 226 is not a sufficient condition for specificity for basic residues. Neither hPRO3 nor hnELA, both of which have an Asp$^{226}$, recognizes a Lys or Arg-P$_1$ residue. The two enzymes display close similarity of their S$_1$ sites and cleave after small mostly hydrophobic residues, such as Leu/Ile (hnELA), Ala/Ser (hPRO3), and Val/Met (hnELA and hPRO3) (38). The presence of Ile and Val at position 190 of hPRO3 and hnELA, respectively, seems partially responsible for their substrate specificities. In hnELA, loss of specificity for basic residues has been attributed to inaccessibility of Asp$^{226}$ that is shielded by Val$^{190}$ and Val$^{216}$. Similarly, Asp$^{226}$ of hPRO3 is also shielded by Ile$^{190}$ and Val$^{216}$. Taken together, the data indicate that Arg/Lys substrate specificity is structurally determined not only by the presence but also by the accessibility of an acidic side chain at the base of the specificity pocket, positioned either at 189 or 226. The carboxyl oxygens of Asp$^{226}$ or Glu$^{226}$ seem less available to substrate than those of Asp$^{189}$ because of participation in hydrogen-bonding networks with residues on the wall of the pocket. This appears to be a distinct feature observed in factor B, the neutrophil elastases, and cCOLL.

Structural and functional consequences of altering the Asp$^{189}$ of trypsin have been examined by site-directed mutagenesis, kinetic, and crystallographic analysis (39). The negative charge was relocated to the opposite wall of the binding pocket in rat trypsin mutant D189G/G226D. Kinetic analysis showed that, compared with wt trypsin, this relocation of the negative charge caused 10$^4$- and 4.5 × 10$^2$-fold decrease in catalytic efficiency ($k_{cat}/K_m$) toward P$_1$-Arg and -Lys containing substrates, respectively. The decrease resulted from a much sharper decline in $k_{cat}$ for the Arg than the Lys substrates, whereas the binding affinity ($K_m$) for both substrates was equally reduced. The crystal structure of D189G/G226D trypsin in complex with inhibitors showed that in its new position, Asp interacts extensively with other residues in the pocket through hydrogen bonds, which greatly reduce its negative charge potential. Similarly to trypsin D189G/G226D, the native Asp$^{226}$ of factor B forms hydrogen bonds and this correlates with the low binding affinity and overall low catalytic efficiency toward P$_1$-Arg/Lys peptide substrates (12–14). Re-constructing the pocket of factor B in the D226N/N189D mutant caused complete loss of hemolytic and C3 proteolytic activity (Figs. 5 and 6), although esterolytic activity toward the P$_1$-Arg thioester substrate was partially retained (Fig. 7). The kinetic analysis showed that the 80% reduction in esterolytic activity ($k_{cat}/K_m$) was almost entirely due to reduction in $k_{cat}$ whereas the $K_m$ was not affected. Thus, the exact location of the negative charge at base of the S$_1$ site and particularly its spatial relationship to the His$^{57}$-Ser$^{195}$ dyad and the oxyanion hole, which is altered in trypsin D189G/G226D and factor B D226N/N189D, are especially critical for efficient catalysis.

In an effort to directly compare factor B to trypsin, a Gly residue was substituted at position 226 either alone (D226G) or in combination with the N189D mutation (D226G/N189D). Neither mutant had hemolytic activity. However, loss of hemolytic activity could not be attributed exclusively to defective substrate recognition at the S$_1$ site because the ability of these mutants to participate in the assembly of the C3 convertase was also affected (Figs. 3 and 5). Binding of the mutants to CoVF and their sensitivity to factor D cleavage was substantially decreased indicating conformational changes near or at the C3b/CoVF-binding sites, which are presumed to be distal to the mutation sites. Because overall folding of the polypeptide chain and the conformation of antigenic epitopes appeared unaffected, the conformational alteration of the C3b-binding site must be subtle, albeit functionally significant. At present it is not clear how the catalytic center relates spatially to the C3b/CoVF-binding sites. Hourcade et al. (30) also described a conformational change at a site distal from the mutation in the F227A mutant (30). The mutant was cleavable by factor D, but cleavage did not promote the conformational change to a high affinity C3b-binding proteolytically active state, which characterizes wt factor B. The Bb fragment of this mutant was recognized by a Bb-specific mAb at much lower efficiency than the wt counterpart. As viewed in the structure of B-SP, the RDFSFIN$^{225–230}$ segment forms an extended internal $\beta$-strand, which is buried within the protein core. Substituting Ala for Phe at position 227 might destabilize the core, affecting the conformation of the surface epitope recognized by the Bb-specific mAb (30). This epitope is probably located near the RDFSFIN$^{225–230}$ segment and is only reactive in Bb perhaps because it is sterically hindered by the Ba region of intact factor B or because it undergoes a conformational change upon cleavage/removal of Ba. Our D226G mutants might have conformational change(s) within the same region. However, the relationship between the possible conformational change of the antigenic epitope and that of the C3b-binding site is still unclear.

It is of interest that the RDFSFIN$^{225–230}$ motif is found in factor B and C2 of most animal species, but is absent from all other complement enzymes (1) as well as from other SPs of the
large chymotrypsin family (40, 38). This underlines the fundamental role of Asp\(^{226}\) in the function of factor B and C2 in complement activation. Therefore, the native conformation of Asp\(^{226}\) and Asn\(^{189}\) or Ser\(^{189}\) within the S1 pocket of factor B and C2, respectively, constitutes one of the structural determinants, which have evolved to optimize the highly specific C3/C5 cleavage. However, C3/C5 recognition and hydrolysis require more extensive enzyme-substrate contacts than interaction of the side chain of P1-Arg with residues of the S1 site. The disparity in catalytic activity toward C3 and dipeptide substrates of N189D and D226N/N189D factor B (Figs. 6 and 7) probably reflects the complexity of the interaction between C3b-bound Bb and its natural substrates, C3 and C5.

In the present study, we correlated the crystal structure of B-SP to the detailed mutational analysis of the factor B S1 C3b-bound Bb and its natural substrates, C3 and C5.

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