Induced Fit Activation Mechanism of the Exceptionally Specific Serine Protease, Complement Factor D†

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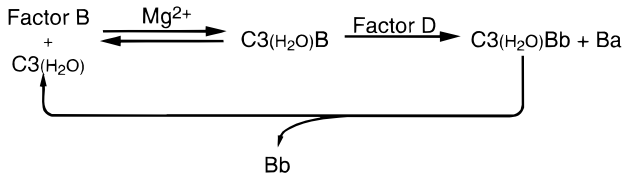
ABSTRACT: We have investigated the mechanism by which the complement protease, Factor D, achieves its high specificity for the cleavage of Factor B in complex with C3(H_2O). Kinetic experiments showed that Factor B and C3(H_2O) associate with a K_D of ≥2.5 μM and that Factor D acts on this complex with a second-order rate constant of $k_{cat}/K_M \geq 2 \times 10^6$ M^{-1} s^{-1}, close to the rate of a diffusion-controlled reaction for proteins of this size. In contrast, Factor D, which is a member of the trypsin family of serine proteases, was 10^3-10^4-fold less active than trypsin toward both thioester and *p*-nitroanilide substrates containing an arginine at P1. Furthermore, peptides spanning the Factor B cleavage site were not detectably cleaved by Factor D ($k_{cat}/K_M \leq 0.5$ M^{-1} s^{-1}). These results imply that contacts between Factor D and the C3(H_2O)B complex, outside the vicinity of the cleavage site in Factor B, generate ≥9 kcal/mol of binding energy to stabilize the transition state for reaction. In support of this, we demonstrate that chemical modification of Factor D at a single lysine residue that is distant from the active site abolishes the activity of the enzyme toward Factor B while not affecting activity toward small synthetic substrates. We propose that Factor D may exemplify a special case of the induced fit mechanism in which the requirement for conformational activation of the enzyme results in a substantial increase in substrate specificity.

Factor D is a 23 kDa serine protease, homologous to serine proteases of the chymotrypsin fold, that catalyzes the initial proteolytic step in the alternative pathway of complement. In this step, Factor D activates its 93 kDa substrate, Factor B, by cleaving between Lys234 and Arg235 (*3*). Factor B is a substrate in this reaction only when in complex with an activated form of the 190 kDa complement protein C3 (*4*), either the hydrolytically activated form C3(H_2O) or the proteolytically activated form C3b (*5*), or with the related snake venom protein cobra venom factor (CVF)[1] (*6*). The cleavage of Factor B results in the release of a ~30 kDa N-terminal fragment, Ba, leaving the ~60 kDa fragment Bb complexed with C3 (Scheme 1). Factor D is exceptionally specific; it has no known protein or peptide substrates other than Factor B in complex with C3 or CVF. The extremely high specificity of Factor D is necessitated by the fact that it circulates in the blood as an active enzyme (*7*), and is not regulated by any known endogenous inhibitor. Factor D displays only low activity toward synthetic protease substrates such as peptide thioesters and *p*-nitroanilides (*8*). The kinetics of its reaction with Factor B have not previously been explored in a quantitative study. The crystal structure of human Factor D (*1*; M. Karpusas et al., unpublished data) shows that the positions of the main chain atoms of Factor D can for the most part be superimposed on those of trypsin. However, significant deviations from the structures observed for trypsin and other serine proteases were found in and around the active site, including striking differences in the side chain conformations of the catalytic histidine and aspartate residues. These observations led to the postulation of an induced fit mechanism for Factor D, in which the catalytically active conformation of the enzyme is achieved only upon binding to the substrate (*1*, *8*). Support for this hypothesis has been provided by observations that mutant forms of Factor D can be generated, with substitutions at sites identified through a comparison of the crystal structures of Factor D and trypsin, that possess an increased activity toward thioester substrates (*9*−*11*; for a review, see ref *12*).

Scheme 1



We describe here a kinetic study of the activity of recombinant human Factor D toward its natural substrate, Factor B in complex with activated C3, in comparison to its

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[1] Abbreviations: *p*NA, *p*-nitroanilide; TBE, thiobenzyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; CHO, chinese hamster ovary; DHFR, dihydrofolate reductase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TFA, trifluoroacetic acid; CVF, cobra venom factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; CM, carboxymethyl; MALDI-TOF, matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry; IEF, isoelectric focusing; NHS, *N*-hydroxysuccinimide.

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activity toward peptides from the Factor B sequence and toward peptide thioesters and *p*-nitroanilides. The results allow us to define the conformational activation of Factor D in more detail and to quantify some of its kinetic and thermodynamic consequences. These results were used to construct a quantitative description of the relationship between the specificity of Factor D and its requirement for conformational activation by its specific substrate, leading to the proposal that Factor D exemplifies a special case of induced fit (*13*) in which, contrary to the general case (*14*), the requirement for conformational activation of the enzyme results in a substantial increase in specificity.

MATERIALS AND METHODS

Materials. Factor B, C3, Properdin, and samples of Factor D for comparison to the recombinant protein were obtained from Quidel (San Diego, CA), as was an ELISA kit for the detection of the Factor B cleavage fragment, Bb. Gradient polyacrylamide gels (4 to 20%) and colloidal Coomassie stain were from Novex (San Diego, CA). Peptide thiobenzyl ester and peptide *p*-nitroanilide substrates were obtained from Enzyme Systems Products (Dublin, CA) and from Bachem (Torrance, CA), respectively. Synthetic peptides were from Research Genetics (Huntsville, AL).

Expression and Purification of Recombinant Factor D. A CHO cell line stably expressing human Factor D was produced by inserting a Factor D syngene into an expression vector driven by tandem SV40 early and adenovirus late promoters and containing a DHFR selectable marker. The syngene sequence contained the natural Factor D signal sequence for cellular export as well as the N-terminal pro region of the Factor D sequence. After purification, the expressed Factor D was found to have a specific activity identical to that of the natural Factor D in both the thioester and Factor B cleavage assays. It should be noted that initial attempts to construct a syngene based on the sequence presented in Figure 1 of White et al. (*15*) yielded a protein with only 2.5% of the specific activity of commercial (Quidel) preparations of human Factor D because of typographical errors in the publication (Ala27[2] should be Gly and Arg34 should be Gln).

To purify Factor D, the conditioned medium was adjusted to pH 6 with 6 M HCl, centrifuged to remove particulates, and applied to a Fast S column (Pharmacia, Piscataway, NJ). The column was washed with 50 mM HEPES buffer (pH 7), and Factor D was eluted with 1 M NaCl. The eluant was then diluted 10-fold with 50 mM MES buffer (pH 5) and applied to a Poros S column (Perseptive Biosystems, Framingham, MA). The column was washed with 50 mM HEPES (pH 7.0) and the Factor D eluted with a gradient of 0 to 1 M NaCl in the HEPES buffer. During this step, the small portion of Factor D in the preparation that retained the zymogen peptide sequence was separated from the fully processed Factor D. Pooled fractions containing Factor D were concentrated and chromatographed on a Sephacryl 100HR column (Pharmacia) in 100 mM HEPES (pH 7.5) containing 500 mM NaCl. The Factor D peak from the gel filtration column was applied to a ceramic hydroxyapatite column (Integrated Separation Systems, Natick, MA), equilibrated with 10 mM sodium phosphate (pH 6.8), and eluted with a 0 to 0.4 M sodium phosphate gradient. Studies with peptide substrates, described below, suggested that very small amounts of contaminating proteases were occasionally present in these preparations. These were eliminated by further purification on a Beckman C18 Ultrapore column with a 0 to 80% acetonitrile gradient in 0.1% TFA/H_2O. Fractions of 0.5 mL were collected in 2 mL of 1 M HEPES (pH 7.5). After dialysis, the specific activity of the Factor D was fully recovered.

Kinetic Measurements. (1) Assay for the Cleavage of Factor B. Reactions were performed by incubating Factor D (0.1−500 nM) with Factor B (0.5−3 μM), C3 (0.2−1.8 μM), and Properdin (0.9 μM) in 100 mM HEPES, 100 mM NaCl, and 1 mM $MgCl_2$ (pH 7.5, 37 °C). Although the C3 was not specifically treated before use to convert it to C3-(H_2O) (a form in which the internal thioester bond is hydrolyzed, rendering it capable of participating in the Factor D reaction; *16*), control experiments involving quantitative analysis of the fraction of C3 that is susceptible to cleavage by CVFBb established that a sizable fraction (typically ∼50%) of the C3 had spontaneously undergone conversion to C3(H_2O) (data not shown), consistent with the known instability of native C3 (*17*). Aliquots of each reaction mixture were removed at various times, and the reactions were quenched by boiling briefly with electrophoresis sample buffer containing 2-mercaptoethanol and SDS. At the end of an experiment, all of the quenched aliquots were run on 4 to 20% SDS polyacrylamide gels and stained with colloidal Coomassie blue. The extent of reaction was determined by densitometry of the stained bands corresponding to unreacted Factor B and to the Bb cleavage fragment, performed using a Molecular Dynamics scanning densitometer. In some experiments, the reaction was followed by an alternative method in which the evolution of the Bb fragment was monitored using a commercial ELISA kit. Properdin was included in the reaction mixtures for reasons related to parallel studies of the reactivity of the C3 convertase; control experiments showed that its inclusion had no detectable effect on the rate of the Factor B cleavage reaction (data not shown).

(2) Reactions with Chromogenic Substrates. Reactions with peptide thiobenzyl ester substrates were performed by adding 5−30 μL of a substrate stock solution and 10 μL of a 50 mM DTNB solution, both in DMSO, to 950 μL of buffer containing, for Factor D, 100 mM HEPES, 1 mM $MgCl_2$, and 1 M NaCl (pH 7.5) or, for trypsin, 100 mM HEPES, 10 mM $CaCl_2$, and 1 M NaCl (pH 7.5, 25 °C). Additional DMSO was added to adjust the reaction mixtures to 4% v/v DMSO, and the solutions were placed in the thermostatically controlled sample compartment of a CARY 3 spectrophotometer and allowed to equilibrate to the reaction temperature of 25 °C for 15 min. Reaction was initiated by adding 10 μL of an enzyme stock solution to give a final enzyme concentration of 46 nM Factor D or 3.5 nM trypsin, and was monitored by the increasing absorbance at 412 nm due to the reaction of the benzyl thiol reaction product with DTNB, using an extinction coefficient of $\epsilon_{412} = 14\,150\ M^{-1}\ cm^{-1}$ for the 2-nitro-5-thiobenzoic acid dianion (*18*). Reaction rates were determined from initial slopes of the progress

[2] Factor D residues are numbered beginning with the first amino acid of the mature protein after removal of the zymogen peptide [i.e., Ile1 in this manuscript is Ile16 in the chymotrypsinogen numbering system of Narayana et al. (*1*)].

curves, and were corrected for the background hydrolysis of the thioester substrates. Separate control experiments were performed to show that DTNB at concentrations up to 1.5 mM had no measurable effect on the activity of Factor D and that the reaction of benzyl thiol with DTNB under these conditions was very fast compared to the cleavage of substrate. The presence of 4% v/v DMSO in the assay was shown to have only a small effect (~15%) on the rate of the reaction. Reactions with peptide *p*-nitroanilide substrates were performed identically, except no DTNB was included in the reactions of *p*-nitroanilide substrates and the concentrations of Factor D and trypsin were 1.56 μM and 35 nM, respectively. Reactions were monitored by following the evolution of *p*-nitroaniline directly, at 405 nm, using an extinction coefficient that was determined under the conditions of the reaction to be $\epsilon_{405} = 10\,400\ M^{-1}\ cm^{-1}$. Background hydrolysis of the *p*-nitroanilide substrates was found to be negligible under the conditions of the experiment. For Factor D, replots of initial velocity versus substrate concentration were linear in all cases, allowing a value for k_{cat}/K_M to be determined from the slope and the known enzyme concentration, but giving only lower limits for k_{cat} and K_M. Similar replots for the reactions of trypsin with the peptide *p*-nitroanilide substrates were hyperbolic, and were fitted to the Michaelis−Menten equation by nonlinear regression analysis using DeltaGraph (DeltaPoint, Inc.).

Assay for the Enzymatic Cleavage of Short Peptides Corresponding to the Cleavage Site in Factor B. Peptides were incubated with either Factor D, trypsin, or buffer without protease, and the reaction products were analyzed by reversed-phase HPLC or capillary electrophoresis. An internal standard peptide, EILDVP, was included for purposes of quantitation. The final concentrations of reaction components were 800 μM test peptide, 877 μM internal standard peptide, and 1.8 μM Factor D or 1.8 nM TPCK-treated trypsin (Sigma), 150 mM NaCl, 1.5 mM $MgCl_2$, and either 100 mM HEPES (pH 7.5) or 100 mM sodium phosphate (pH 7.5). After incubation at 37 °C for 18 h, the enzyme was removed from the reaction mixture by ultrafiltration through a 5000 MW cutoff filter and the filtrate frozen at −80 °C until chromatographic analysis. Reversed-phase HPLC analysis was carried out on a Beckman C18 Ultrapore column with a 0 to 80% acetonitrile gradient in 0.1% TFA/H_2O, monitored at 215 nm. Capillary electrophoresis was carried out on a 50 μm inside diameter, 57 cm capillary cartridge on a Beckman P/ACE 2200 system at 30 kV with a running buffer of 20% acetonitrile in 100 mM phosphate (pH 2.5), monitored at 215 nm. In some experiments, a mixture of protease inhibitors, shown in control experiments not to inhibit Factor D, was included to avoid proteolysis by contaminating proteases. The protease inhibitors were AEBSF, aprotinin, leupeptin, pepstatin, E64, benzamidine, and EDTA.

Preparation of Monobiotinylated Factor D. Factor D (1 mg) was reacted with an equimolar amount of sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in 1 mL of 0.1 M sodium bicarbonate buffer (pH 8.5) at ambient temperature for 1 h. The reaction was terminated by adding 10 mM ethanolamine. The biotinylated species were separated from unmodified Factor D by chromatography on a CM-POROS column (PerSeptive Biosystems, Framingham, MA) with a 0 to 0.5 M gradient of NaCl in 50 mM MES buffer (pH 6.5). One new Factor D species was found that eluted just prior to the unmodified Factor D.

Peptide Mapping. Samples containing about 20 μg of Factor D (0.5 mg/mL) were reduced in 0.1 M Tris-HCl, 1 mM EDTA, and 6 M guanidine hydrochloride (pH 8.5) with 40 mM DTT at ambient temperature overnight, after which 0.5 μL of 4-vinylpyridine was added and the solution was kept at ambient temperature for 2 h. The alkylated proteins were recovered by precipitation with 40 volumes of cold ethanol (*19*). The solution was stored at −20 °C for 1 h and then centrifuged at 14000*g* for 6 min at 4 °C. The supernatant was discarded, and the precipitate was washed once with cold ethanol. The alkylated proteins were dissolved in 1 M guanidine hydrochloride with 0.1 M Tris-HCl (pH 8.0) and digested with 1:10 (w/w) endoproteinase Lys-C (Wako Pure Chemical Industries, Ltd.) at ambient temperature for 20 h. Digests were analyzed by reversed-phase HPLC using a Waters 2690 Alliance Separations Module with a model 996 photodiode array detector (Waters Corp.). A 2.1 mm × 25 cm Vydac C4 column was used with a 50 min gradient (0 to 80% acetonitrile) in 0.1% trifluoroacetic acid at a flow rate of 0.2 mL/min. Individual peaks were collected for further analysis. Biotinylated peptide 181−209 was further digested with endoproteinase Glu-C (Boehringer Mannheim GmbH) at a 1:20 (w/w) ratio in 50 mM NH_4HCO_3 (pH 8.0) at ambient temperature for 18 h. The peptides were then separated by reversed-phase HPLC using a 2.1 mm × 25 cm Vydac C18 column as described above.

Analysis of Peptides by Mass Spectrometry. Molecular ion masses of peptides were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy on a Voyager-DE STR (PerSeptive Biosystems). α-Cyano-4-hydroxycinnamic acid was used as the matrix. Sequencing of the biotinylated peptide 195−209 was performed on the same mass spectrometer by post-source decay measurement.

CD Analysis. Measurements were taken on a JASCO model J-715 spectropolarimeter. Spectra were taken at 20 °C in a 1 mm path length cell. The concentrations of the proteins were 0.16 mg/mL in 10 mM phosphate (pH 7.2) with 150 mM NaCl.

RESULTS

Activity of Factor D toward Thioester and p-Nitroanilide Substrates. It has previously been shown that Factor D possesses extremely low activity toward activated peptide substrates such as peptide *p*-nitroanilides (*20*) and thioesters (*8*). We have explored the activity of Factor D toward a group of synthetic substrates, including a direct comparison of the reactivities of Factor D and of trypsin toward some of the same substrates, to better quantify this aspect of the reactivity of the enzyme. Figure 1 shows that Factor D catalyzes the hydrolysis of the highly reactive thioester substrate, (*Z*)-Arg-Arg-thiobenzyl ester, at a rate that is linearly dependent on the concentration of substrate up to its solubility limit in the reaction buffer. The data give the following values for the kinetic constants: $k_{cat}/K_M = (2.6 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$, $K_M \geq 85\ \mu M$, and $k_{cat} \geq 0.2\ s^{-1}$, consistent with the low activity of Factor D toward other thioester substrates (*8*). The open circles in Figure 1 show that omission of the 1 M NaCl from the reaction buffer lowered k_{cat}/K_M by ~60%. We also tested

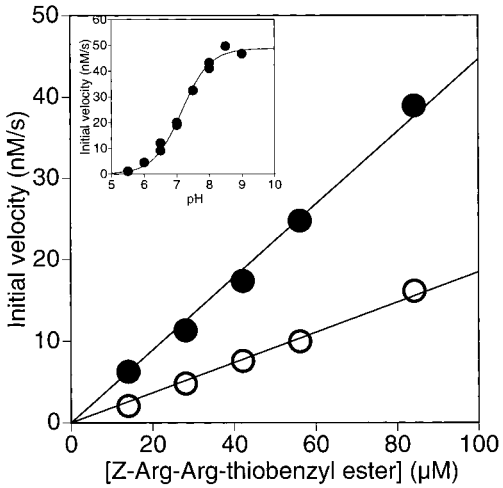

FIGURE 1: Activity of Factor D toward (*Z*)-Arg-Arg-thiobenzyl ester at high and low salt concentrations [175 nM Factor D, 100 mM HEPES, 1 mM $MgCl_2$, 0.5 mM DTNB, 4% v/v DMSO at pH 7.5 and 25 °C with 0 (●) or 1 M (○) NaCl]. The reaction was monitored at 412 nm by following the rapid reaction of the benzyl thiol reaction product with DTNB. The inset plot shows the initial velocity of the Factor D-catalyzed hydrolysis of (*Z*)-Arg-Arg-thiobenzyl ester as a function of pH.

the pH dependence of the initial reaction velocity at a fixed, subsaturating substrate concentration (Figure 1, inset), which corresponds to the pH dependence of k_{cat}/K_M. The rate decreased with pH over the range of 9.0−5.5, with an inflection at pH 7.2 suggesting that a catalytically important residue on the free enzyme ionizes with a pK_a of ∼7.2. A pH of 7.5 was chosen for subsequent kinetic measurements involving Factor D, because at this pH the background hydrolysis of the substrate is relatively slow and yet the enzyme activity is ∼70% of the maximum rate seen at high pH. In other experiments, it was shown that the presence of DTNB in the reaction buffer had no effect on the enzyme activity over the range of 0.25−1.5 mM, and that the introduction of 4% v/v DMSO with the substrate reduced the reaction rate by only ∼15% (data not shown). Other peptide thiobenzyl esters were tested as substrates, and several that contain arginine or lysine at P1 were found to be moderately good substrates for the enzyme (data not shown), consistent with the results seen for a wide range of peptide thioester substrates by Kam et al. (*8*). The close agreement between our rate constants and those measured by Kam et al., for those substrates that were tested in both studies, establishes that the recombinant enzyme used in this work is equivalent in activity to the natural enzyme.

To quantitatively compare the activities of Factor D and trypsin toward small synthetic substrates, we measured the kinetics of the reactions of both enzymes with a group of substrates encompassing variations both in the length of the peptide acyl group and in the nature of the leaving group (Table 1). Substrates with peptide portions containing Arg or Lys-Arg were chosen to match the residues found at P1 and P2[3] in the cleavage site in Factor B (*3*). The data in Table 1 show that k_{cat}/K_M values for reaction of Factor D with (*Z*)-Lys-Arg-*p*NA and (*Z*)-Lys-Arg-TBE are smaller than those for the corresponding reactions of trypsin by a factor of 3−4 × 10^3; the smaller substrates, (*Z*)-Arg-*p*NA and (*Z*)-Arg-TBE, were cleaved 1−2 × 10^4-fold more slowly by Factor D than by trypsin. Thus, although this group of substrates encompasses a ∼10^5-fold range of absolute reactivities, the relative reactivities of trypsin versus those of Factor D are all similar to within a factor of ∼7. Our results agree well with data obtained by others using thioester substrates only (*8*, *11*).

[3] Nomenclature of Schecter and Berger (*2*).

Table 1: Comparative Activities of Factor D and Trypsin toward Peptide Thioesters and *p*-Nitroanilides

substrate		k_{cat}/K_M (M^{-1} s^{-1})		
acyl group	leaving group	Factor D[a]	trypsin[b]	activity ratio of trypsin/factor D
(*Z*)-Lys-Arg	benzyl thiol	2100[c]	6800000[c]	3200
(*Z*)-Arg	benzyl thiol	350 ± 50	3000000 ± 30000	8600
(*Z*)-Lys-Arg	*p*-nitroaniline	0.62 ± 0.05	2700 ± 300	4400
(*Z*)-Arg	*p*-nitroaniline	0.019 ± 0.003	430 ± 50	23000

[a] In 0.1 M HEPES, 1 mM $MgCl_2$, 1 M NaCl, 0.5 mM DTNB, and 4% v/v DMSO at pH 7.5 and 25 °C. [b] In 0.1 M HEPES, 10 mM $CaCl_2$, 1 M NaCl, and 4% DMSO at pH 7.5 and 25 °C. [c] From Kam et al. (*8*).

Activity of Factor D toward Factor B. The activity of Factor D toward Factor B was investigated by incubating Factor B with Factor D in the presence of C3 and the stabilizing factor Properdin (100 mM HEPES, 100 mM NaCl, and 1 mM $MgCl_2$ at pH 7.5 and 37 °C). It was shown in separate experiments that ∼50% of the C3 used in these experiments was typically in the form C3(H_2O) that supports Factor B cleavage (*16*) (see Materials and Methods). The progress of Factor B cleavage was monitored by SDS−PAGE or by ELISA as described in Materials and Methods. Figure 2A shows that when the reaction is performed using a concentration of total C3 (0.56 μM) that is roughly similar to that of Factor B (0.54 μM), the initial velocity of the reaction appears to be linearly dependent on the concentration of Factor D. This result shows that the Factor D-dependent step is rate-determining under these conditions; the reaction rate is not being limited by the rate of association of B with C3(H_2O) to form the C3(H_2O)B substrate complex, or by any other Factor D-independent process (Scheme 1). Similar measurements, taken at a concentration of total C3 (53 nM) that is substoichiometric in relation to the Factor B concentration of 1.1 μM, showed a hyperbolic dependence of the reaction rate on the concentration of Factor D (Figure 2B). This is because, at limiting levels of C3, increasing the concentration of Factor D to a sufficient level causes the rate of the Factor D-catalyzed proteolytic cleavage step to become fast compared to that of the dissociation of C3(H_2O)-Bb, causing the recycling of C3 to become rate-limiting (Scheme 1). Figure 3 shows that, at a total C3 concentration of 0.53 μM, the initial reaction velocity is roughly proportional to the concentration of Factor B over the range of 0.55−2.76 μM. Increasing the concentration of Factor B at a fixed concentration of C3 increases the reaction rate by increasing the concentration of the substrate complex, C3-(H_2O)B. The linear dependence of the initial rate on the concentration of Factor B indicates that the association of B and C3 to form C3(H_2O)B must be quite weak (K_D ≥ 2.5 μM). If the association of these two proteins were stronger than this, then the fraction of C3 in complex with B would be or become saturated as the concentration of B increased over this range, leading to an invariance or a hyperbolic dependence of the rate as a function of the concentration of B. We can infer from the

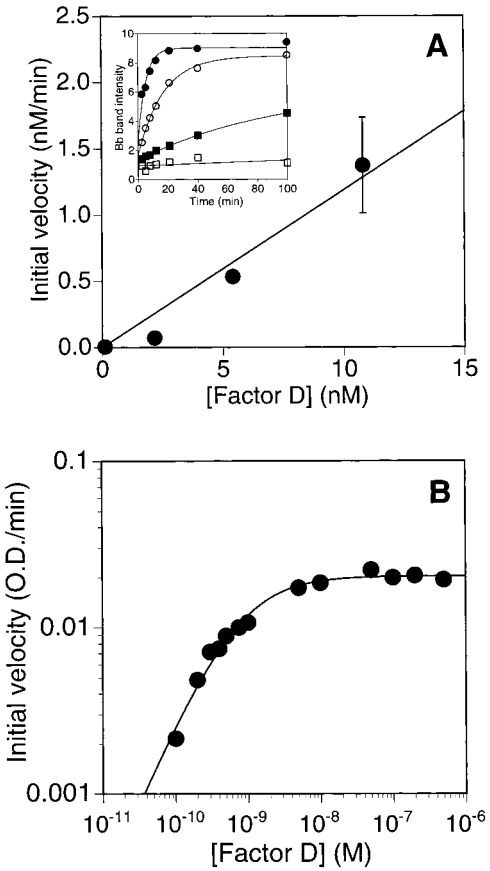

FIGURE 2: Initial velocity of C3(H_2O)B cleavage as a function of Factor D concentration at high (A) and low (B) concentrations of total C3. (A) Factor D (0.11−10.8 nM) was incubated with a 1:1 ratio of Factor B (0.54 μM) and C3 [0.56 μM, estimated to be ~50% C3(H_2O)] in 100 mM HEPES containing 0.9 μM Properdin, 100 mM NaCl, and 1 mM $MgCl_2$ at pH 7.5 and 37 °C. Aliquots of each reaction mixture were removed at various times and the reactions quenched by boiling briefly with electrophoresis sample buffer containing 2-mercaptoethanol and SDS. Quenched aliquots were separated on 4 to 20% SDS polyacrylamide gels and stained with colloidal Coomassie blue. The extent of reaction was determined by densitometry of the stained bands corresponding to unreacted Factor B and to the Bb cleavage fragment. The inset plot shows time courses for the evolution of Bb band intensity at 10.8, 5.4, 2.16, and 0.11 nM Factor D; the solid lines represent the best fits of the data to an exponential equation. (B) Factor D (0.1−500 nM) was incubated with a 20:1 ratio of Factor B (1.1 μM) and C3 [53 nM, estimated to be ~50% C3(H_2O)] in 100 mM HEPES and 1 mM $MgCl_2$ at pH 7.5 and 37 °C. Aliquots of each reaction mixture were removed at 0, 15, 30, and 45 min, and the extent of reaction at each time point was determined by measuring the concentration of the Bb cleavage fragment using a commercial ELISA kit (see Materials and Methods). Data are plotted as a log−log plot of initial velocity vs Factor D concentration. The data were fitted to the equation for a rectangular hyperbola by nonlinear regression analysis, as shown by the solid line.

absence of saturation that [C3(H_2O)B] $\leq {}^1/_2$[B]$_{total}$, even at the highest concentration of Factor B used (2.76 μM); thus, we can set an upper limit to the concentration of C3(H_2O)B present in any given reaction. The initial rate of Factor B cleavage was also linearly dependent on the concentration of total C3 over the C3 concentration range of 0.018−1.8 μM (data not shown), consistent with the K_D value of ≥2.5 μM for the interaction of Factor B with C3(H_2O) that was calculated from the data in Figure 3. This limit for the interaction affinity between B and C3(H_2O) is in agreement with the low micromolar value of K_D reported by Pryzdial

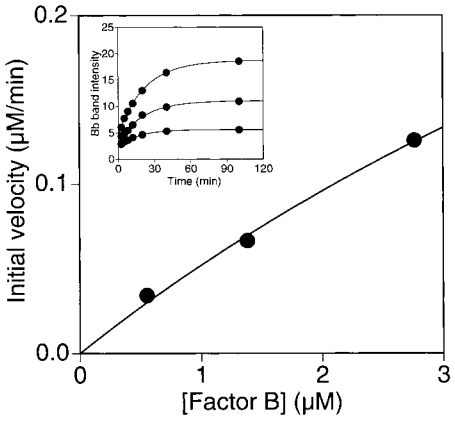

FIGURE 3: Initial velocity of C3(H_2O)B cleavage by Factor D as a function of the concentration of Factor B. Factor D (2.16 nM) was incubated with Factor B (0.54−2.76 μM) and C3 (0.56 μM) in 100 mM HEPES containing 0.9 μM Properdin, 100 mM NaCl, and 1 mM $MgCl_2$ at pH 7.5 and 37 °C. The reaction was followed as described in the legend of Figure 2A. The inset plot shows time courses for the evolution of Bb band intensity at 0.54, 1.38, and 2.76 μM Factor B; the solid lines represent the best fits of the data to an exponential equation.

and Isenman (*21*) based on equilibrium binding studies. In addition to establishing a lower limit for the mutual affinity of Factor B and C3(H_2O), the data in Figure 3 show that the concentrations of C3(H_2O)B that are achieved under these conditions are low relative to K_M for the turnover of this substrate, since linear relationships rather than saturation kinetics are seen. This conclusion is also apparent from the exponential form of the progress curves such as those shown in Figure 3. Failure to approach saturation of the rate with respect to substrate concentration clearly precludes obtaining explicit values for k_{cat} or K_M for the cleavage of C3(H_2O)B by Factor D. Moreover, because we have only an upper limit for the concentration of C3(H_2O)B under any particular set of conditions, we can calculate only a limiting value for k_{cat}/K_M of $\geq 2 \times 10^6$ M^{-1} s^{-1}, obtained from the slope of the best-fit line in Figure 3 assuming that the limit to the true substrate concentration is given by [C3(H_2O)B] $\leq {}^1/_2$[B]$_{total}$. Similarly, only a lower limit for k_{cat} of ≥ 5 s^{-1} can be calculated. Importantly, even this lower limit to the true value for k_{cat}/K_M is already close to the diffusional encounter rate for proteins of the size of Factor D (23 kDa) and C3(H_2O)B (270 kDa) (*14*). When considered in terms of k_{cat}/K_M, the cleavage of C3(H_2O)B by Factor D is thus a very fast reaction.

C3-Dependent Cleavage of Factor B by Trypsin and Plasmin. Published reports strongly suggest that the requirement that Factor B bind to C3 to become reactive as a substrate for Factor D is, at least in part, the consequence of a large conformational change in Factor B that unmasks the site of cleavage (*7*). Direct contacts between Factor D and C3(H_2O) may also contribute to enhancing the reaction rate, analogous to the mechanisms of certain cofactor proteins in the blood coagulation system. It has not been shown whether the increased reactivity of the cleavage site in Factor B that accompanies the conformational change arises from its unmasking and exposure as a flexible strand or instead from its presentation in a strained or unusual conformation that renders it specifically complementary to the active site of Factor D. To distinguish between these possibilities, we examined the proteolytic cleavage of Factor B by four

Table 2: Site of Cleavage of Factor B by Factor D, Plasmin, and Trypsin[a]

enzyme	concentration (nM)	% reaction[a] C3	% reaction[a] no C3	cleavage site in sequence QK↓R↓K↓IV K↓R	R↓K	K↓I
Factor D	2.8	85	0	0	100	0
plasmin	2200	55	16	~33	~33	~33
trypsin	5.6	30	4	~50	~50	0

[a] [B] = 0.1 mg/mL. [C3] = 0 or 50 μg/mL. The enzyme concentration is indicated. 100 mM HEPES, 75 mM NaCl, and 1 mM $MgCl_2$ at pH 7.5 and 37 °C for 30 min.

proteases, Factor D, trypsin, plasmin, and EndoArg, in the presence and absence of 0.5 μM C3. Product mixtures were analyzed by SDS−PAGE, and the cleaved products were subjected to N-terminal sequencing in each case to establish the exact site of cleavage by the different enzymes. Table 2 shows that, as expected, the cleavage of Factor B (1.1 μM) by Factor D (2.8 nM) absolutely requires the presence of C3, and occurs exclusively at the previously established cleavage site after Arg234 (*3*). Under the same conditions, trypsin (5.6 nM) and plasmin (2.2 μM) were also found to cleave Factor B at or near the Factor D cleavage site, as has been previously reported for trypsin (*3*, *22*). The data indicate that trypsin and Factor D catalyze the reaction with almost equal efficiency. The reactions with trypsin and plasmin are significantly accelerated by C3 but do not absolutely require its presence, consistent with C3 increasing the exposure of the cleavage site within Factor B. In contrast to Factor D, these two enzymes show no preference for cleavage at Arg234 over other adjacent basic sites. EndoArg at 2.2 μM gave no reaction in either the presence or the absence of C3. Plasmin and trypsin are known to bind substrates and peptide inhibitors in a β-strand conformation (*23*), and to prefer substrates that have this conformation or are flexible enough to adopt it without strain. Therefore, the fact that these two enzymes can efficiently cleave Factor B, and particularly that they do so with no selectivity for the specific Factor D site over adjacent basic sites, argues strongly against the cleavage site in Factor B being presented in any highly strained or unusual conformation that is peculiarly complementary to the active site of Factor D. The data instead suggest that binding to C3 causes the exposure of the cleavage site in Factor B as a flexible loop or strand. In addition to product bands migrating at molecular masses of about 60 and 30 kDa, corresponding to the expected Bb and Ba fragments, both trypsin and plasmin showed an additional minor product that ran slightly below Bb on gels; similar product distributions were seen in the absence of C3 despite the lowered extent of reaction. Sequencing showed that these minor products have the same N-terminal sequences as those found for the ~60 kDa Bb band, indicating that these fragments arise from cleavage at one or more additional sites fairly close to the C-terminus of Factor B.

Activity of Factor D toward Peptides Derived from the Factor B Cleavage Site. For many proteases, the reactivity of substrates is strongly dependent on the binding of amino acid residues extending for several positions on each side of the scissile bond, to specific subsites in the extended active site cleft of the enzyme (*23*). To test for the existence of critical contacts involving substrate residues close to the scissile bond in Factor B, we tested as substrates peptides derived from the sequence spanning the cleavage site in Factor B. Peptides of varying lengths were selected: KRKIV, QKRKIV, and PGEQQKRKIVLDP, as well as the cyclic peptide CKRKIVC. The longest of these represents the portion of Factor B extending across the cleavage site from P7 to P6′.[3] The peptides were tested as substrates under conditions similar to those used for the Factor D-dependent cleavage of Factor B. The extent of cleavage that occurred upon incubation with 1.8 μM Factor D for up to 18 h at 37 °C was determined by HPLC and capillary electrophoretic analysis. In no case was more than 15% of the substrate peptide cleaved during the reaction. Furthermore, no specific cleavage products could be detected by HPLC, suggesting that even the small loss of substrate peptides that was observed in certain cases might be due to the action of trace contaminating proteases other than Factor D. The Factor D used in these initial tests with peptide substrates was purified through the penultimate (hydroxyapatite) step of the purification protocol, but had not been subjected to the final HPLC purification. This result was therefore confirmed by repeating the experiment with the longest and apparently most active of the peptides, PGEQQKRKIVLDP, using Factor D that had been purified through the final reversed-phase chromatographic step (shown to give fully active enzyme), or which had been treated with a mixture of protease inhibitors that had been found not to inhibit Factor D activity toward Factor B or peptide thiobenzyl ester substrates (see Materials and Methods). Under these conditions, no cleavage of the peptide whatsoever (i.e., <5%) was observed, whereas a 1000-fold lower concentration of TPCK-treated trypsin was shown to fully cleave the test peptides. The same series of peptides was tested for binding to the active site of Factor D, by determining whether they inhibit the Factor D-catalyzed hydrolysis of the thioester substrate (*Z*)-Arg-Arg-thiobenzyl ester. No inhibition was seen at peptide concentrations up to 2 mM. Since the thioester substrate was present at subsaturating concentrations, the failure of the peptides to inhibit the enzyme implies that their affinity for binding to the active site of Factor D has a limit of $K_D \geq 2$ mM. The failure of peptides derived from the cleavage site of Factor B to inhibit the activity of Factor D toward thioester substrates is not consistent with some published reports (*24*, *25*). The reason for this discrepancy is not known, though it might be explained if the Factor D used in these published experiments was contaminated with trace amounts of other proteases. From the absence of detectable cleavage of the substrate peptides upon incubation with Factor D, we estimate that Factor D cleaves even the longest of these Factor B-derived peptides with a k_{cat}/K_M of ≤ 0.5 M^{-1} s^{-1}. This number is $>10^6$-fold lower than the k_{cat}/K_M for the cleavage of the same amino acid sequence within the context of C3(H_2O)B.

We were surprised that Factor D showed so little binding affinity for peptides with sequences identical to that of the cleavage site of Factor B, a sequence that must occupy the active site of Factor D when the enzyme is acting upon C3(H_2O)B. We therefore explored the apparent inability of peptides based upon this sequence to bind to the active site of the enzyme by testing variants of the N- and C-terminal protected cleavage site-derived hexapeptide, Ac-QKRKIV-NH_2 (Table 3), as inhibitors of the reaction with a thioester substrate. When tested at concentrations of 1 mM, none of

Table 3: Variants of Factor B Cleavage Site Peptides Tested as Inhibitors of Factor D

series 1	series 2	series 3
Ac-**A**KRKIV-NH_2	Ac-QKR-NH_2	Ac-R**G**I-NH_2
Ac-Q**A**RKIV-NH_2	Ac-KRK-NH_2	Ac-R**G**IV-NH_2
Ac-QK**A**KIV-NH_2	Ac-RKI-NH_2	Ac-RK**G**V-NH_2
Ac-QKR**A**IV-NH_2	Ac-KIV-NH_2	
Ac-QKRK**A**V-NH_2		
Ac-QKRKI**A**-NH_2		

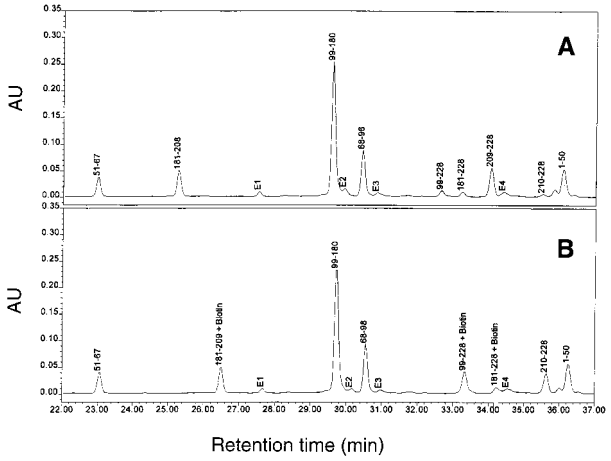

FIGURE 4: Peptide map of unmodified (A) and biotinylated (B) Factor D. Factor D was modified by the lysine-specific reagent sulfo-NHS-LC-biotin and separated from unmodified protein by cation-exchange chromatography. The proteins were then reduced, alkylated with 4-vinylpyridine, and digested with the lysine-specific protease endoproteinase Lys-C. The resulting peptides were separated by reversed-phase chromatography and identified by MALDI-TOF mass spectrometry. The numbers over each peak indicate the amino acid sequence segment from which the peptide was derived. Peaks marked with E are peptides from autodigestion of endoproteinase Lys-C. The corresponding mass ions are given in Table 4.

the peptides in series 1, 2, or 3 showed any inhibition, indicating that their affinity for binding to the active site of Factor D has a limit of $K_D > 1$ mM. Thus, the failure of peptides derived from the cleavage site in Factor B to bind in the active site of Factor D does not appear to be due to any isolated unfavorable contacts with specific residues in the peptides, but rather suggests a more general lack of complementarity between the peptides and the resting state of the enzyme active site.

Blocking of Factor B Cleavage by Biotinylation or Acetylation of Factor D at a Site Distant from the Active Site Cleft. Reaction of Factor D with an equimolar concentration of the biotinylation reagent sulfo-NHS-LC-biotin followed by purification of the modified enzyme, as described in Materials and Methods, gave a product that was shown by MALDI-TOF mass spectrometry to contain a single biotin label. IEF electrophoresis showed a single new band, suggesting the possibility that Factor D was biotinylated at a single site. This was confirmed by MALDI-TOF and post-source decay analysis of endoproteinase Lys-C peptide digests (Figures 4 and 5 and Table 4). The peptide map of unmodified Factor D includes a peptide at *m*/*z* 2870.44 corresponding to amino acids Gly181−Lys208 and a peptide at *m*/*z* 2181.17 corresponding to amino acids Lys209−Ala228, demonstrating cleavage after Lys208 is favored over cleavage at Lys209, presumably because of the proline at position 210. However, in the map of the

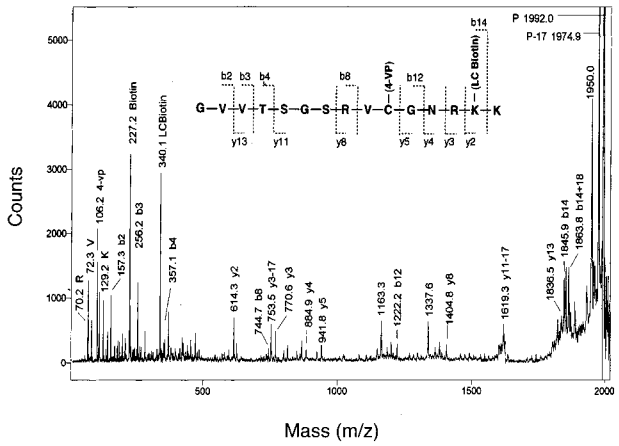

FIGURE 5: Post-source decay mass spectrometry sequencing of the biotinylated peptide of residues 181−209. The biotinylated peptide of residues 181−209 from the endoproteinase Lys-C digest shown in Figure 4 was collected and further digested with endoproteinase Glu-C to generate the peptide of residues 195−209 for analysis by PSD. The nomenclature is as follows. y fragments originate from the C-terminus, and b fragments originate from the N-terminus.

Table 4: Mass Spectrometry Analysis of Peptides from Unmodified and Biotinylated Factor D[a]

peptide	MH^+ (Da) calculated	MH^+ (Da) measured
residues from endo Lys-C digest		
1−50	5398.18	5399.4
51−67	1789.67	1789.9
68−98	3596.91	3596.7
99−180	9498.94	9503.3
181−208	2870.44	2870.6
181−209 and biotin	3337.70	3337.9
209−228	2181.17	2181.4
210−228	2053.08	2053.2
181−228	5035.81	5036.7
181−228 and biotin	5374.98	5374.3
99−228	14515.72	14519.5
99−228 and biotin	14854.89	14861.2
residues from endo Glu-C digest		
181−194	1364.65	1364.5
195−209 and biotin	1992.06	1992.0

[a] Reversed-phase peptide map shown in Figure 4.

monobiotinylated material, neither of these peptides was found. Instead, a new ion at *m*/*z* 3337.70 appeared that corresponds to peptide Gly181−Lys209 plus the addition of one biotin. The map of biotinylated Factor D also showed increased amounts of peptide Pro210−Ala228 and the partial cleavage products Ala99−Ala228 and Gly181−Lys228 (plus biotin). The other peptides in the maps were also identified by MALDI-TOF and remained unchanged after biotinylation. These data demonstrate that the unique biotinylation site is Lys208 [Lys223*A* using the chymotrypsinogen numbering system (*1*)], the middle lysine in a basic triplet RKK, which becomes protected from cleavage by endoproteinase Lys-C. This site of biotinylation was confirmed by collecting peptide Gly181−Lys209, further digesting it with endoproteinase Glu-C, and subjecting the product peptide Gly195−Lys209 to PSD analysis (Figure 5). The critical mass ions found were b14 (parent peptide minus the C-terminal lysine) and y2 (final two C-terminal amino acids). Biotinylation at Lys208 was confirmed since both these fragments contained the biotin adduct. Preferential biotinylation of this site by the NHS

reagent may be due to an increase in the deprotonated fraction of the ϵ-amine as a consequence of the flanking positively charged residues.

The enzymatic activity of the purified, monobiotinylated Factor D was compared to that of unmodified Factor D (recovered from the same biotinylation reaction mixture by CM-POROS chromatography) toward (*Z*)-Arg-Arg-thiobenzyl ester and also toward C3(H_2O)B. No difference was found in the rate of hydrolysis of the thioester substrate, but the rate of cleavage of Factor B was only about 1% of the activity of the unmodified enzyme. The same result was found when NHS-acetate was substituted for NHS-biotin, showing that a less bulky modification of the enzyme also results in selective inhibition of the productive binding of Factor D to the C3(H_2O)B complex. The circular dichroism spectra of unmodified and biotinylated Factor D were superimposable (data not shown), indicating no large modification of the structure of the enzyme resulted from biotinylation.

DISCUSSION

Published reports have included the suggestion that the apparently very high substrate specificity displayed by Factor D might be a consequence of an induced fit activation mechanism in which binding of the substrate induces the enzyme to adopt a more reactive conformation (*8*, *12*). The crystal structure of Factor D (*1*), and subsequent mutagenesis studies (*9*−*11*), provide strong evidence that conformational reorganization of the active site and its environs is indeed required for the enzyme to achieve full activity. However, without kinetic data on the activity of Factor D toward its physiological substrate, Factor B in complex with C3, it has not previously been possible to develop a quantitative understanding of how the requirement for conformational activation by the substrate might translate into increased specificity for Factor B over other potential substrates. This question is of general importance since there are conflicting views on the precise circumstances under which an induced fit mechanism can contribute to increasing the specificity of an enzyme (*13*, *14*, *26*).

Direct comparison of the activities of Factor D and trypsin toward the peptide *p*-nitroanilide and thioester substrates in Table 1 shows a large and essentially invariant k_{cat}/K_M ratio for the two enzymes of 10^3-10^4. Although this comparison includes only a small number of substrates, these were selected because, like Factor B, they contain Arg or Lys-Arg in the positions immediately upstream of the scissile bond, and therefore should interact optimally with the corresponding subsites in the active site of Factor D. The observed variations in acyl group structures and leaving groups within the series result in absolute reactivities that cover a range of $\sim 10^5$, so the fact that the relative reactivities of these substrates for hydrolysis by trypsin compared to Factor D are identical to within a factor of ~ 7 is notable. This result indicates that the energy required to reach the transition state for the acylation step (i.e., the first irreversible step) in the hydrolysis of these substrates, from the ground state of free enzyme plus free substrate, is larger for Factor D than for trypsin by a constant difference of ~ 5 kcal/mol. For Factor D, this activation barrier includes the energy required to go from the inactive resting conformation of the enzyme to what is presumably an active conformation in the transition state for reaction. The additional ~ 5 kcal/mol required to reach the transition state for Factor D may, therefore, provide a rough estimate of the energy difference between the resting and active conformations of the enzyme. This interpretation is supported by the data of Kim et al. (*9*−*11*), which show that a series of mutations in Factor D that were designed to favor a more "trypsin-like" conformation at the active site results in an increase of up to 100-fold in the activity of the enzyme toward thioester substrates. Our data suggest that the total increase in activity toward such substrates that would be achieved by the full conformational activation of Factor D may be substantially larger still.

To place the low activity of Factor D toward small reactive substrates in context, it is necessary to know how these numbers compare to the activity of the enzyme toward its specific substrate, Factor B in complex with an activated form of C3. Our results establish that C3(H_2O)B is cleaved very efficiently by Factor D, with a k_{cat}/K_M of $\geq 2 \times 10^6$ M^{-1} s^{-1}. This rate is close to the diffusional encounter rate for two molecules of the size of Factor D and C3(H_2O)B, and is comparable in magnitude to rate constants for the association steps of other enzyme reactions, especially those involving proteins as substrates (*14*). The diffusional encounter rate defines the theoretical maximum rate constant for a bimolecular reaction, and has been used as a benchmark for identifying evolutionarily "perfect" enzymes (*27*). Thus, even the lower limit to k_{cat}/K_M that we were able to determine for the reaction of C3(H_2O)B establishes that Factor D cleaves its specific substrate very rapidly indeed.

Information about which specific features of the C3(H_2O)B complex are responsible for its high reactivity as a substrate was provided by looking at the properties of Factor B-derived peptides as substrates and as inhibitors of Factor D. Peptides containing an amino acid sequence identical to that spanning the cleavage site in Factor B are very poor substrates; k_{cat}/K_M is $>10^6$ times lower than that for cleavage of the same sequence of amino acids within C3(H_2O)B. The longest of these peptides, the 13-mer PGEQQKRKIVLDP, extends across the cleavage site from P_7 to $P_6'^3$, and is thus certainly long enough to interact with all of the extended binding subsites that exist in the active site of Factor D. Thus, the structural determinants on the substrate that are responsible for the high specificity of the enzyme for Factor B lie outside the immediate vicinity of the cleavage site that binds in the active site cleft of Factor D. In addition to their failure to react as substrates for Factor D, even the longest of the Factor B-derived peptides failed to bind detectably to the active site of the enzyme ($K_D > 2$ mM). No single residue appears to be responsible for the failure to bind to the enzyme; even when cleavage site hexapeptides in which each residue in turn was replaced with alanine or combinations of residues were omitted entirely were used, we failed to identify a variant with detectable affinity for the active site. These binding data indicate a striking lack of complementarity between the substrate sequence and the active site of the enzyme in its resting conformation, consistent with an induced fit mechanism.

The fact that trypsin and plasmin readily proteolyze the cleavage strand in Factor B, and the lack of selectivity of these enzymes for cleaving at the precise site at which Factor D acts compared to other adjacent basic sites, provides strong

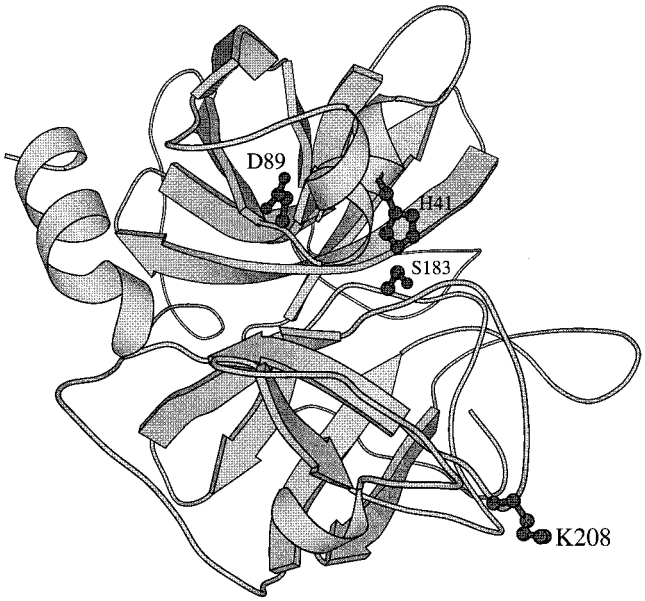

FIGURE 6: Representation of the crystal structure of Factor D showing the location of Lys208 (Lys223*A* in the chymotrypsinogen numbering system) with respect to the catalytic residues His41 (His57), Asp89 (Asp102), and Ser183 (Ser195) in the active site. Lys208 is a solvent-exposed residue located ~18 Å from the active site serine in a direction perpendicular to the active site cleft. The figure was prepared from the coordinates of Narayana et al. (*1*) using the program MolScript 2.1 (*28*).

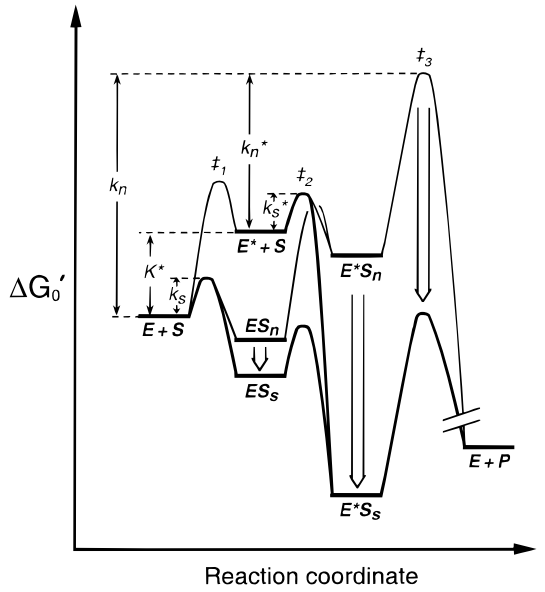

FIGURE 7: Hypothetical free energy profiles illustrating how the proposed induced fit mechanism would result in increased specificity for cleavage of C3(H_2O)B over nonspecific substrates. The upper curves (drawn with thin lines) show that reaction with a nonspecific substrate, S_n, which is unable to selectively stabilize the active conformation of the enzyme, E*, will proceed through rate-limiting conformational ($‡_2$) or chemical ($‡_3$) steps. For the reaction of S_n with E, $k_{cat}/K_M = k_n$. For reaction of S_n with a hypothetical constitutively active form of the enzyme resembling E*, $k_{cat}/K_M = k_n$*, giving a reaction that is faster by a factor of K* which is equal to the equilibrium constant for the conversion of E to E*. The lower curve (thick lines) shows how specific complementarity between C3(H_2O)B (S_s) and E* stabilizes the reactive Michaelis complex E*S and the ground and transition states for subsequent steps of the reaction. As a result of this stabilization, for reaction of S_s with E, the association step ($‡_1$) is rate-limiting and $k_{cat}/K_M = k_s$. For reaction with a hypothetical constitutively active form of the enzyme resembling E*, association of S_n with E* is rate-limiting and $k_{cat}/K_M = k_s$*. Because both k_s and k_s* represent association events, the reaction of S_s with either E or E* is diffusion-controlled and, unlike the situation with S_n, k_{cat}/K_M is not decreased by the need for E to undergo transformation to E*. The broad arrows represent the magnitude of the binding energy generated by distal site interactions between Factor D and C3(H_2O)B: small in ES due to imperfect complementarity with the inactive conformation E, larger in $‡_2$ in which the conformational activation of E is taking place, and full in E*S and $‡_3$ in which Factor D exists in its active conformation and can thus fully realize the interaction energy available through distal site binding interactions with C3(H_2O)B.

evidence that the specificity of Factor D for C3(H_2O)B over peptides with similar sequences is not due to presentation of the cleavage site in Factor B in a strained or unusual conformation. Instead, we interpret the $>10^6$-fold higher activity of Factor D for C3(H_2O)B, compared to those for the peptide substrates, in terms of a requirement for specific binding interactions between Factor D and distal sites on the C3(H_2O)B substrate complex that are absent in the peptide substrates. The observed ratio of $>10^6$ in k_{cat}/K_M implies that the binding energy generated by these distal site interactions contributes ≥ 9 kcal/mol to the stabilization of the rate-limiting transition state. Direct evidence for the existence of distal site interactions between Factor D and its specific substrate is provided by the selective inhibitory effect of biotinylating or acetylating the enzyme at Lys208 [Lys223*A* using the chymotrypsinogen numbering system (*1*)]. Figure 6 shows that Lys208 is a solvent-exposed residue located ~18 Å from the active site serine in a direction perpendicular to the active site cleft. Circular dichroism measurements established that biotinylation at this site did not cause any gross change in the protein's structure. Moreover, the absence of any effect on the activity of Factor D toward a thioester substrate shows that the modification does not affect the intrinsic catalytic activity of the enzyme. The almost complete abolition of activity against Factor B is therefore interpreted in terms of the disruption of critical interactions between Factor D and C3(H_2O)B that involves Lys208 or its environs. Our results do not allow us to distinguish whether these crystal site interactions involve contacts with Factor B, with C3, or with both components of the substrate complex.

Fersht has pointed out that an induced fit mechanism does not, in general, confer an increased substrate specificity on an enzyme, because it reduces the activity of the enzyme toward both good and poor substrates by an identical factor corresponding to the difference in free energy between the resting and active conformations of the enzyme (*14*). However, Herschlag has raised the theoretical possibility of special cases of induced fit that would give rise to an increase in substrate specificity (*13*). We propose that one of these special cases may apply to Factor D. The high specificity of Factor D can be directly linked to its requirement for a substrate-induced conformational activation if the rate-determining step in the reaction with C3(H_2O)B is assumed to be the diffusional encounter between the inactive form of Factor D and the substrate, as the high k_{cat}/K_M observed for this reaction suggests might be the case, but if the reaction with nonspecific substrates is limited by the rate of chemical or conformational steps occurring after the diffusional encounter step. Hypothetical free energy profiles for the reaction of specific and nonspecific substrates according to this proposal are illustrated in Figure 7. The upper curves (drawn with thin lines) in Figure 7 shows that for reaction

with a nonspecific substrate S_n, for which the chemical steps of the reaction ($\ddagger_3$) are rate-limiting, the activation barrier that governs k_{cat}/K_M is given by k_n. A nonspecific substrate is defined as a substrate that does not selectively bind and stabilize the active form of the enzyme (E*), compared to the conformationally inactive resting form (E). The rate at which S_n would be cleaved by a hypothetical, constitutively active form of the molecule can be seen by considering E* + S as the ground state; in this case, k_{cat}/K_M corresponds to the activation barrier k_n*. Thus, as has been discussed by others (*13*, *14*), the existence of a conformationally inactive form of the enzyme decreases k_{cat}/K_M for reaction of a nonspecific substrate by a factor of K*, corresponding to the equilibrium constant for the conversion of E to E*. We propose that specific binding interactions between Factor D and C3(H_2O)B, the distal site interactions established by our data, may generate sufficient binding energy to stabilize complexes containing E* such that transition states $\ddagger_2$ and $\ddagger_3$ for the steps after formation of ES become lower in energy than $\ddagger_1$. The lower curve (thick line) in Figure 7 illustrates the consequences of this proposal. If $\ddagger_2$ and $\ddagger_3$ are stabilized sufficiently to bring them below $\ddagger_1$, ES will partition predominantly in the forward direction. Thus, k_{cat}/K_M will equal k_s, the rate constant for the initial encounter step, leading to the observation of diffusion-controlled kinetics. The rate at which the specific substrate S_s would be cleaved by a hypothetical, constitutively active form of the enzyme can again be seen by considering E* + S as the ground state; for an equivalent degree of stabilization of $\ddagger_2$ and $\ddagger_3$, k_{cat}/K_M is given by the rate constant for the association event, k_s*, and therefore will also be diffusion-controlled. Thus, the effect of the enzyme existing in a conformationally inactive resting state is to destabilize all ground and transition states that contain E* by a factor of K*. For a substrate that generates sufficient binding energy through specific interactions with the active form of the enzyme to compensate for this effect, and stabilize these states such that $\ddagger_2$ and $\ddagger_3$ are brought below $\ddagger_1$, the fact that the enzyme exists in an inactive resting state has no effect on k_{cat}/K_M, which is diffusion-controlled in either case. By contrast, the effect of an inactive resting state for the enzyme on the reaction of a nonspecific substrate, which is unable to make sufficient selective binding interactions with E*, is to lower k_{cat}/K_M by a factor of K*. The existence of an inactive resting state for the enzyme thus increases the specificity of the enzyme for S_s over S_n by an additional factor of K* above the specificity that would be manifested by a hypothetical constitutively active form of the enzyme. This is a consequence of the fact that only the specific substrate has sufficient complementarity for E* to ensure that conformational activation of the enzyme occurs *after* the rate-limiting transition state. For nonspecific substrates, conversion from E to E* precedes the rate-limiting step, and thus, the equilibrium constant for this process, K*, appears in the denominator of the expression for k_{cat}/K_M. For Factor D, we estimate that K* is on the order of 10^3-10^4, suggesting the additional specificity that Factor D achieves from the existence of an inactive resting state is considerable.

In summary, the very high specificity of Factor D, which is required for its role as initiator of the alternative pathway of complement, originates from a very highly evolved and specific complementarity between the active conformation of Factor D and the reactive conformation of Factor B in complex with C3(H_2O). We propose that this complementarity is translated into specificity for C3(H_2O)B by means of a special case of induced fit which, contrary to the general case, provides an additional large specificity factor as a direct result of the requirement for substrate-induced conformational activation of the enzyme. Although the variation on the induced fit mechanism described above has been postulated on theoretical grounds by Herschlag (*13*), to our knowledge Factor D is the first enzyme for which there is experimental evidence to support the operation of such a mechanism. This proposal provides a potential explanation for the observation of a conformationally inactive structure for Factor D in crystallographic studies (*1*; M. Karpusas et al., unpublished data) and may explain how it is that Factor D can circulate as a mature enzyme, without leading to inappropriate proteolysis of endogenous proteins.

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