Allosteric Effects of a Monoclonal Antibody against Thrombin Exosite II†

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ABSTRACT: We previously isolated a monoclonal antithrombin IgG from a patient with multiple myeloma [Colwell et al. (1997) *Br. J. Haematol. 97*, 219−226]. Using a panel of 55 surface mutants of recombinant thrombin, we now show that the epitope for the IgG most likely includes Arg-101, Arg-233, and Lys-236 in exosite II. The IgG affects the rate at which thrombin cleaves various peptide *p*-nitroanilide substrates with arginine in the P1 position, increasing the k_{cat} for substrates with a P2 glycine residue but generally decreasing the k_{cat} for substrates with a P2 proline. The allosteric effect of the IgG is altered by deletion of Pro-60b, Pro-60c, and Trp-60d from the 60-loop of thrombin, which lies between exosite II and the catalytic triad. The effect of the IgG, however, does not depend on the presence or absence of sodium ions, a known allosteric regulator of thrombin. The IgG does not affect the conformation of thrombin exosite I as determined by binding of a fluorescent derivative of hirudin$^{54-65}$. These results provide evidence for a direct allosteric linkage between exosite II and the catalytic site of thrombin.

Thrombin is the final proteolytic enzyme generated in the blood coagulation cascade. Its procoagulant functions include activation of platelets (*1*), conversion of fibrinogen to fibrin, and activation of factors V, VIII, XI, and XIII (*2*−*5*). Thrombin also inhibits fibrinolysis by activation of a plasma carboxypeptidase (*6*) and exerts an anticoagulant effect by activation of protein C (*7*). The substrate specificity of thrombin is more limited, however, than that of many other serine proteases such as trypsin. A distinctive feature of thrombin is the presence of unique insertion loops at positions 60 and 148 (chymotrypsinogen numbering), which border the active site cleft and restrict access of macromolecular substrates and inhibitors to the catalytic serine residue (*8*−*10*). Thrombin also possesses two patches of positively charged amino acid residues, called anion-binding exosites I and II, which reside at some distance from the active site and facilitate interaction with other macromolecules. For example, exosite I binds fibrin(ogen), the thrombin receptor, thrombomodulin, hirudin, and heparin cofactor II, whereas exosite II binds glycosaminoglycans and prothrombin fragment 2 (*11*, *12*). Alterations in the catalytic activity of thrombin can be detected after binding of thrombomodulin or other ligands to exosite I (*13*−*15*). Binding of ligands to exosite II of thrombin also produces a conformational change that can be detected with active site-directed fluorescence probes (*16*, *17*) or functional assays (*18*−*21*). Furthermore, binding of a sodium ion to a single site converts thrombin from a form that preferentially cleaves protein C to a form that preferentially cleaves fibrinogen (*22*, *23*). Thus, thrombin is an allosteric enzyme.

Recently, we identified a monoclonal antithrombin IgG in the plasma of a patient with multiple myeloma and a severe bleeding disorder (*24*). The IgG bound to human α-, β-, and γ-thrombin but not to prothrombin, other vitamin K-dependent coagulation factors, or fibrinogen. At concentrations ≥25 nM, the purified IgG caused a 50% decrease in the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA[1] by thrombin. Even after prolonged incubation with >6 μM IgG, thrombin retained ∼50% of its amidolytic activity, indicating that the partial inhibition observed was not due to slow binding of the monoclonal IgG to thrombin. In the present study, we demonstrate that the monoclonal IgG, which most likely binds to Arg-233 in exosite II of thrombin, alters the kinetics of hydrolysis of a variety of synthetic peptide substrates. Furthermore, we show that the allosteric effect of the IgG depends on the presence of the 60-loop, which resides on the surface of thrombin between the active site and exosite II, but does not depend on the presence or absence of sodium ions. In addition, the IgG does not affect binding of a fluorescent analogue of hirudin to exosite I. These results suggest a direct allosteric linkage between exosite II and the catalytic site of thrombin.

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[1] Abbreviations: pNA, *p*-nitroanilide; thrombin(desPPW), recombinant thrombin with deletion of Pro-60b, Pro-60c, and Trp-60d; TSP buffer, Tris-buffered saline containing poly(ethylene glycol); [5F]Hir$^{54-65}$, hirudin$^{54-65}$ labeled at the amino terminus with 5-carboxyfluorescein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials. Human α-thrombin was purchased from Haematologic Technologies (Essex Junction, VT). Thrombin-(desPPW) (*10*) was kindly supplied by Dr. Charles T. Esmon, Oklahoma Medical Research Foundation, Oklahoma City. The molar concentration of thrombin(desPPW) was determined by titration of its amidolytic activity with hirudin (Sigma, St. Louis, MO) using human α-thrombin as the standard. Chromogenic substrates were purchased from the following sources: S-2765, S-2222, S-2366, S-2238, and S-2288 from DiaPharma Group (Franklin, OH); spectrozyme FXa, spectrozyme FIXa, spectrozyme PCa, spectrozyme TH, and spectrozyme Pro from American Diagnostica (Greenwich, CT); and chromozym TH from Boehringer Mannheim (Indianapolis, IN). Substrate concentrations were determined by absorbance at 342 nm (E = 8270 $M^{-1}\cdot cm^{-1}$) (*25*). Porcine intestinal heparin (grade I-A, 183 USP units/mg) was obtained from Sigma.

Expression and Activation of Recombinant Prothrombin Variants. Cos-7 cells were transiently transfected with wild-type and mutant prothrombin cDNAs in the pRc/CMV vector (Invitrogen, San Diego, CA) as previously reported (*26*). Serum-free conditioned medium (~2 mL) was harvested after 36 h and concentrated to 50−100 μL in a Centricon-30 ultrafiltration device (Amicon, Beverly, MA). The concentrated medium was incubated for 30 min at 37 °C with 5 μL of 0.35 μg/μL *Echis carinatus* snake venom (Sigma) to convert the prothrombin to thrombin. The venom was pretreated with 20 mM 4-amidinophenylmethanesulfonyl fluoride (Sigma) to inactivate contaminating serine proteases (*27*) and was dialyzed into 150 mM NaCl, 50 mM Tris-HCl, and 1 mg/mL poly(ethylene glycol) 8000, pH7.4 (TSP buffer).

Preparation of IgG and Fab Fragments. Purification and characterization of the monoclonal IgG was performed as described previously (*24*). Normal human IgG was purchased from Sigma. Fab fragments were produced by digestion of the IgG (1−10 mg/mL) with activated papain (1 mg of enzyme/100 mg of substrate) for 1 h at 37 °C in 100 mM Tris-HCl, pH 8.0, containing 2 mM EDTA and 1 mM dithiothreitol (*28*). The digestion was stopped by incubation with 20 mM iodoacetamide for 1 h at 4 °C in the dark. The products were dialyzed against phosphate-buffered saline, intact IgG and the Fc fragments were removed by absorption to protein A-Sepharose, and the Fab fragments were further purified by chromatography on Sephacryl S-200. The purity of the final preparation was assessed by SDS−PAGE. The concentrations of the IgG and Fab preparations were determined by absorbance at 280 nm (E = 1.35 $mg^{-1}\cdot mL\cdot cm^{-1}$), assuming molecular weights of 150 000 and 50 000, respectively.

Immunoprecipitation of Recombinant Thrombin. Protein A−Sepharose CL-4B beads (Sigma) were coated with purified monoclonal or normal human IgG as previously described (*24*). A 100 μL sample of Cos-7 conditioned medium treated with *E. carinatus* venom was incubated with 20 μL of beads for 1 h at room temperature with continuous mixing. The beads were then removed by centrifugation. To determine the unbound thrombin activity, the supernatant solution was mixed with 150 μL of 100 μM tosyl-Gly-Pro-Arg-pNA in TSP buffer, and the rate of change of absorbance at 405 nm was determined using a Vmax kinetic microplate reader with Softmax software (Molecular Devices, Sunnyvale, CA). The supernatant activity after immunoprecipitation with the monoclonal IgG is expressed as a percentage of the activity of a duplicate sample incubated with beads coated with normal human IgG.

Activity of Recombinant Thrombin Variants in the Presence of IgG and Heparin. Recombinant thrombin and serial dilutions of the monoclonal IgG (0−19 000 nM) were mixed in 100 μL of TSP buffer at room temperature in a disposable polystyrene cuvette. Tosyl-Gly-Pro-Arg-pNA (500 μL) (100 μM) in TSP buffer was then added, and the rate of change of absorbance at 405 nm was determined. In some experiments, wild-type recombinant thrombin was mixed with the IgG (80 nM) and serial dilutions of heparin (0−20 mg/mL) before addition of the chromogenic substrate.

Effect of the Fab on Hydrolysis of Chromogenic Substrates by Thrombin. Human α-thrombin (10 nM) or thrombin-(desPPW) (55 nM) and serial dilutions of Fab fragments (0−3000 nM) were preincubated in 100 μL of TSP buffer for 1 min at room temperature in a disposable polystyrene cuvette. A chromogenic substrate in TSP buffer (500 μL) was then added, and the rate of change of absorbance at 405 nm was determined. The substrates used and their final concentrations in the 600 μL reaction are listed in Table 1 or in the figure legends. In experiments to investigate the effect of sodium ions, the TSP buffer was replaced by 5 mM Tris-HCl and 1 mg/mL poly(ethylene glycol) 8000, pH 8.0, containing either 200 mM NaCl or choline chloride (*22*).

Steady-State Kinetic Analyses. Thrombin (5 or 8 nM) was preincubated with or without the monoclonal IgG (530 nM) for 1 min at 37 °C in 200 μL of TSP buffer. Prewarmed tosyl-Gly-Pro-Arg-pNA or MeO-CO-D-Chg-Gly-Arg-pNA in TSP buffer (800 μL) was then added to yield the final substrate concentrations indicated, and the initial velocity of substrate hydrolysis was determined by measurement of the absorbance at 405 nm. The extinction coefficient of the product *p*-nitroaniline was assumed to be 9920 $M^{-1}\cdot cm^{-1}$ (*29*). The final thrombin concentrations ([E]) were 1.0 and 1.6 nM in the experiments with tosyl-Gly-Pro-Arg-pNA and MeO-CO-D-Chg-Gly-Arg-pNA, respectively. The data were fit to the Michaelis−Menten equation to determine the K_m and V_{max} values, using Scientist software (MicroMath Scientific Software, Salt Lake City, UT). The k_{cat} values were calculated from the equation $k_{cat} = V_{max}/[E]$.

Binding of [*5F*]*Hir*$^{54-65}$ *to Thrombin*. The fluorescent derivative of Tyr-63-sulfated hirudin dodecapeptide ([5F]Hir$^{54-65}$) was prepared as described previously (*30*). [5F]Hir$^{54-65}$ (12.6 nM) was titrated with α-thrombin (0−600 nM) in the absence or presence of the monoclonal IgG (2 or 4 μM) at 25 °C in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, and 1 mg/mL poly(ethylene glycol) 8000, pH 7.4. Fluorescence measurements were made at excitation and emission wavelengths of 491 and 515 nm, respectively. The results are expressed as the fractional change in the initial fluorescence ($\Delta F/F_0$) and were fit by the quadratic binding equation to determine the maximum fluorescence change ($\Delta F_{max}/F_0$) and dissociation constant (K_D) with one binding site on thrombin assumed for the peptide (*30*).

Table 1: Effect of Monoclonal Fab Fragments on Substrate Hydrolysis[a]

substrate	formula	K_m (μM)	final [substrate] (μM)	rate of hydrolysis: control ($\Delta A405$/min)	rate of hydrolysis: +Fab (%)
S-2765	Cbo-D-Arg-Gly-Arg-pNA		89	0.144	295
S-2222	Bz-Ile-Glu-Gly-Arg-pNA	56[b]	131	0.00079	275
spectrozyme FXa	MeO-CO-D-Chg-Gly-Arg-pNA	52[c]	119	0.020	205
S-2366	pyroGlu-Pro-Arg-pNA	38[b]	62	0.078	166
spectrozyme FIXa	H-D-Leu-Ph′Gly-Arg-pNA		86	0.020	142
chromozym TH	tosyl-Gly-Pro-Arg-pNA	10[c]	98	0.145	50
S-2238	H-D-Phe-Pip-Arg-pNA	3[c]	105	0.092	30
S-2288	H-D-Ile-Pro-Arg-pNA	2[c]	89	0.069	27
spectrozyme PCa	H-D-(γ-Cbo)Lys-Pro-Arg-pNA	5[c]	108	0.061	26
spectrozyme TH	H-D-Hht-Ala-Arg-pNA	3[c]	112	0.041	24
spectrozyme Pro	H-D-Chg-Pro-Arg-pNA	2[c]	101	0.024	21

[a] Thrombin (1.7 nM) and synthetic peptide substrates at the final concentrations indicated were incubated in 600 μL TSP buffer in the absence (control) or presence (+Fab) of monoclonal Fab fragments. The initial rate of hydrolysis of the substrate in the absence of the Fab is indicated (ΔA405/min). The maximum effect of the Fab is expressed as a percentage of the corresponding control value and is taken from the data in Figure 3. K_m values are from the literature. Abbreviations: Bz, benzoyl; Cbo, carbobenzoxy; Chg, cyclohexylglycyl; CO, carbonyl; Hht, hexahydrotyrosyl; MeO, methoxy; Ph′Gly, phenylglycyl; Pip, L-pipecolyl; pNA, *p*-nitroanilide. [b] Data are taken from ref *29*. [c] Data are taken from ref *10*.

RESULTS

Identification of the Epitope on Thrombin for the Monoclonal Human IgG. We expressed a panel of 55 prothrombin variants in Cos-7 cells and activated the recombinant prothrombins to thrombin with *E. carinatus* venom. The reaction mixtures were incubated with protein A−Sepharose beads coated with the monoclonal antithrombin IgG. The beads were then removed by centrifugation, and the supernatant solutions were assayed for thrombin activity (Figure 1). Only two of the thrombin variants were not immunoprecipitated by the monoclonal IgG (solid bars). One of these variants contained a single amino acid substitution (R233A, chymotrypsinogen numbering system), while the other contained three mutations (R233A, K236A, and Q239A).[2] Variants containing either of the two single amino acid substitutions K236A or Q239A, however, were at least partially immunoprecipitated by the monoclonal IgG. These data suggest that the IgG most likely binds to Arg-233 in exosite II of thrombin.

Several mutations, including R101A and K236A, appeared to decrease the affinity of thrombin for the IgG without completely eliminating its binding (Figure 1). To estimate the relative affinities of these mutants for the IgG, we took advantage of the observation that binding of the IgG to thrombin decreases the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA (*24*). Figure 2A compares the rates of hydrolysis of tosyl-Gly-Pro-Arg-pNA by wild-type thrombin and several thrombin variants in the presence of increasing concentrations of the monoclonal IgG. The concentration of IgG that produced half-maximal inhibition (IC_{50}) of wild-type recombinant thrombin was 7 nM. In decreasing order, the IC_{50}s for the thrombin variants tested were as follows: R233A, >18 000 nM; R101A, 4500 nM; K236A, 2000 nM; R93A/R97A/E97aA, 19 nM; E164A/K169A/D170A, 12 nM; and D125A/R126A/E127A, 10 nM. These results suggest that Arg-101 and Lys-236, which are in close approximation to Arg-233 in exosite II, may also be part of the epitope for the IgG.

Arg-101, Arg-233, and Lys-236 have been implicated in binding of heparin to thrombin (*31*−*35*). Figure 2B demonstrates that heparin almost completely reversed the effect of 80 nM monoclonal IgG on hydrolysis of tosyl-Gly-Pro-Arg-pNA. Control experiments indicated that heparin at the highest concentration tested had no effect on the rate of hydrolysis of this substrate in the absence of the IgG (data not shown). These results suggest that heparin competes with the monoclonal IgG for binding to thrombin, consistent with the hypothesis that the epitope for the IgG includes Arg-101, Arg-233, and Lys-236.

Allosteric Effect of the Monoclonal Fab Fragments. Figure 3 shows the effect of Fab fragments prepared from the monoclonal IgG on hydrolysis of a variety of peptide *p*-nitroanilide substrates. Since the control rates of hydrolysis of these substrates by thrombin varied (Table 1), the amidolytic activities shown in Figure 3 were normalized to the values obtained in the absence of the Fab. For some substrates the rate of hydrolysis was increased by the Fab (Figure 3A), while for others it was diminished (Figure 3B). In each case, the rate of hydrolysis approached a plateau at Fab concentrations ≥100 nM, and the half-maximal effect occurred at ∼20−40 nM Fab. We did not identify any substrate for which the amidolytic activity of thrombin was unaltered in the presence of the monoclonal Fab. In control experiments, the monoclonal Fab (3 μM) did not affect the rate of hydrolysis of any of the substrates by trypsin, nor did Fab fragments derived from normal human IgG (3 μM) affect the rate of hydrolysis of any of the substrates by thrombin (data not shown). These results rule out nonspecific interactions of the Fab with the enzyme or substrate.

The experiments shown in Figure 3 were performed at substrate concentrations that, in most cases, were well above reported K_m values for thrombin (Table 1). Therefore, changes in the rate of hydrolysis in the presence of the Fab reflect differences in the turnover number (k_{cat}) of thrombin. We performed a more detailed kinetic analysis of the effect of a saturating concentration of the monoclonal IgG on hydrolysis of tosyl-Gly-Pro-Arg-pNA and MeO-CO-D-Chg-

[2] When this panel of mutants was originally screened for activity (*26*), the triple mutant R233A/K236A/Q239A was reported to be inactive, contrary to the results presented here. Reexamination of the sequences of the thrombin mutants revealed that, by mistake, the activity reported for mutant 38 (K240A/D243A/Q244A) was actually that of mutant 37 (R233A/K236A/Q239A). Thus, the triple mutant R233A/K236A/Q239A retains activity toward chromogenic substrates.

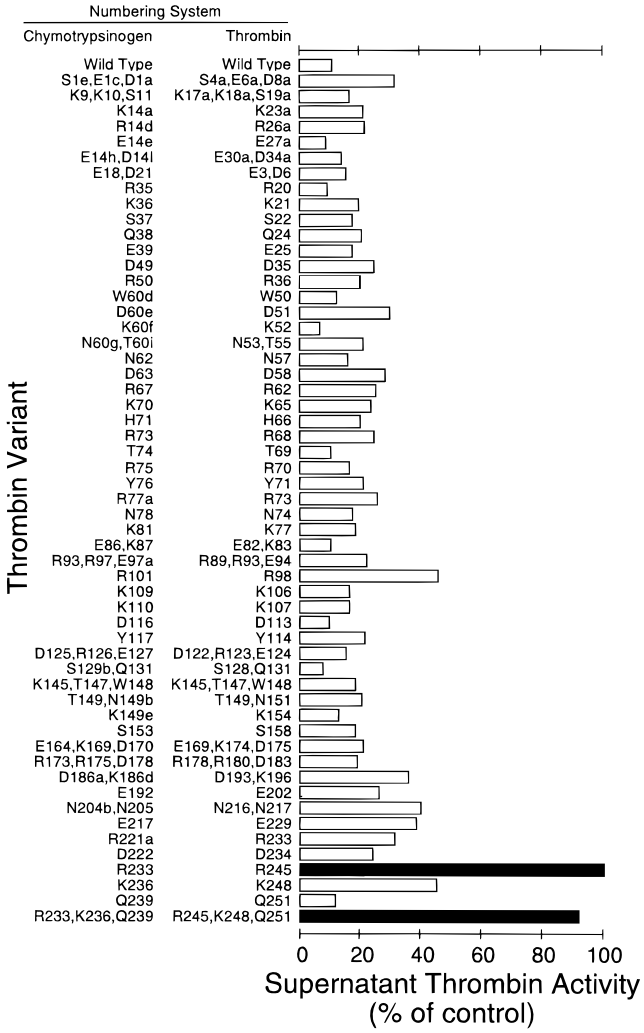

FIGURE 1: Immunoprecipitation of thrombin variants. Mutant prothrombins containing alanine substitutions at the positions indicated were expressed in Cos-7 cells and activated with *E. carinatus* venom. Samples of the activation mixtures were immunoprecipitated with the monoclonal IgG bound to protein A−Sepharose, and the supernatant thrombin activity was determined by hydrolysis of tosyl-Gly-Pro-Arg-pNA. The data are expressed as percentages of the supernatant thrombin activity after control immunoprecipitations with normal human IgG. The solid bars indicate variants that did not bind appreciably to the monoclonal IgG. Thrombin residues are numbered here and in the text according to their topologic equivalence with chymotrypsinogen, as suggested by Bode et al. (*8*). The corresponding positions numbered from the N-terminal ends of the thrombin A-chain (indicated by a in the thrombin column) and B-chain are also shown.

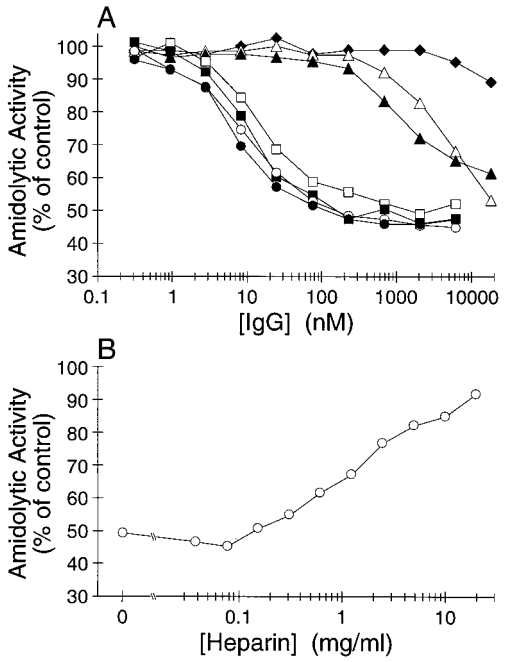

FIGURE 2: Activity of recombinant thrombin variants in the presence of monoclonal IgG or heparin. Panel A: Recombinant thrombin was mixed with monoclonal IgG (0−19 000 nM) in 100 μL of TSP buffer at room temperature. 500 μL of tosyl-Gly-Pro-Arg-pNA (100 μM) in TSP buffer was then added, and the absorbance at 405 nm was recorded continuously. The amidolytic activity is expressed as a percentage of the control $\Delta A405$/min observed in the absence of the IgG, which ranged from 0.072 to 0.141 with various thrombin preparations. Curves: ●, wild type; ○, D125A/R126A/E127A; ■, E164A/K169A/D170A; □, R93A/R97A/E97aA; ▲, K236A; △, R101A; ◆, R233A. Panel B: Wild-type recombinant thrombin was mixed with the monoclonal IgG (80 nM) and heparin (0−20 mg/mL) in 100 μL of TSP buffer at room temperature. Amidolytic activity was then determined as described above. Activity is expressed as a percentage of the control $\Delta A405$/min observed in the absence of IgG and heparin (control $\Delta A405/\text{min} = 0.073$).

Gly-Arg-pNA. The initial velocity of hydrolysis was determined at various concentrations of each substrate in the presence or absence of the IgG (Figure 4), and the data were fit to the Michaelis−Menten equation to estimate the K_m and k_{cat} values (Table 2). The IgG decreased both the K_m and the k_{cat} for tosyl-Gly-Pro-Arg-pNA, resulting in a 2-fold increase in the selectivity (k_{cat}/K_m) of the enzyme for this substrate. By contrast, the IgG increased the k_{cat} for MeO-CO-D-Chg-Gly-Arg-pNA. Although the K_m for the latter substrate appeared to be decreased slightly, the difference from the control value was within the range of experimental error. Similar results were obtained with the monoclonal Fab (data not shown).

Allosteric Effect of the Monoclonal Fab on the Activity of Thrombin(*desPPW*). To investigate the possibility that the 60-loop mediates the effects of the monoclonal IgG on thrombin activity, we used thrombin(desPPW), in which residues Pro-60b, Pro-60c, and Trp-60d were deleted (*10*). In a preliminary experiment, we found that thrombin(desPPW) bound to the immobilized monoclonal IgG, although it eluted at a lower salt concentration (0.8 M NaCl) in comparison with native thrombin (1.0 M NaCl) (data not shown). Figure 5 shows the effects of the monoclonal Fab on hydrolysis of two substrates by thrombin(desPPW). The rate of hydrolysis of MeO-CO-D-Chg-Gly-Arg-pNA by thrombin(desPPW) increased to 220% of the control value in the presence of the Fab, a result similar to that obtained with native thrombin (Figure 3A and Table 1). Unexpectedly, the Fab produced an effect on the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA by thrombin(desPPW) opposite to that of native thrombin. The rate of hydrolysis of this substrate by native thrombin decreased to 50% of the control value in the presence of the Fab (Figure 3B and Table 1), while the rate of hydrolysis by thrombin(desPPW) increased to 194%. The rate of hydrolysis of each of these substrates by thrombin(desPPW) approached a plateau at Fab concentrations ≥400 nM, and the half-maximal effect occurred at ∼200 nM Fab. Therefore, the Fab appears to have a 5−10-fold lower affinity for thrombin(desPPW) in comparison with native thrombin.

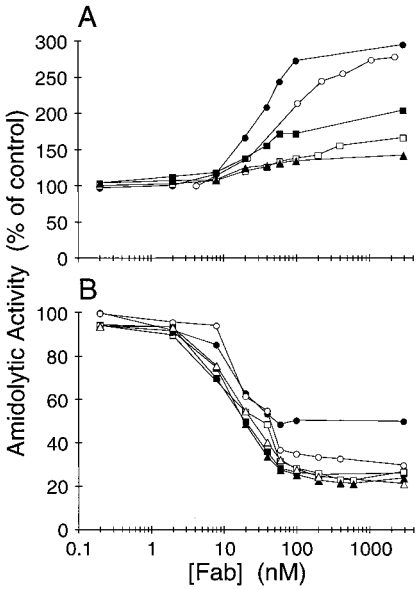

FIGURE 3: Effects of the monoclonal Fab on chromogenic substrate hydrolysis. Thrombin (10 nM) was preincubated for 1 min at room temperature in 100 μL of TSP buffer containing 0−3000 nM Fab fragments prepared from the monoclonal IgG. Substrate (500 μL in TSP) was then added to yield the final concentration indicated in Table 1, and the absorbance at 405 nm was recorded continuously. The amidolytic activity is expressed as a percentage of the rate of hydrolysis of the substrate (ΔA405/min) observed in the absence of the Fab (control values in Table 1). Panel A: ●, Cbo-D-Arg-Gly-Arg-pNA; ○, Bz-Ile-Glu-Gly-Arg-pNA; ■, MeO-CO-D-Chg-Gly-Arg-pNA; □, pyroGlu-Pro-Arg-pNA; ▲, H-D-Leu-Ph′Gly-Arg-pNA. Panel B: ●, tosyl-Gly-Pro-Arg-pNA; ○, H-D-Phe-Pip-Arg-pNA; ■, H-D-Ile-Pro-Arg-pNA; □, H-D-(γ-Cbo)Lys-Pro-Arg-pNA; ▲, H-D-Hht-Ala-Arg-pNA; △, H-D-Chg-Pro-Arg-pNA.

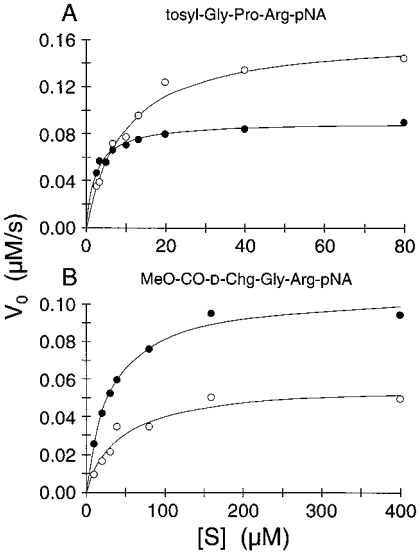

FIGURE 4: Steady-state kinetics. Thrombin (5 nM in panel A, 8 nM in panel B) was preincubated in the absence (○) or presence (●) of the monoclonal IgG (530 nM) for 1 min at 37 °C in 200 μL of TSP buffer. Tosyl-Gly-Pro-Arg-pNA or MeO-CO-D-Chg-Gly-Arg-pNA was then added in 800 μL of TSP buffer to achieve the final concentrations indicated ([*S*]), and the initial velocity of substrate hydrolysis (V_0) was determined by measurement of the absorbance at 405 nm.

Role of Sodium Ions in the Allosteric Effect of the Monoclonal Fab. To determine if the allosteric effect of the monoclonal Fab depends on sodium ions, we compared the effects of the Fab on hydrolysis of substrates in the presence of 200 mM sodium chloride or 200 mM choline chloride (Figure 6). In control incubations without Fab, the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA in choline chloride was ∼40% of the rate in sodium chloride, while the rate of hydrolysis of MeO-CO-D-Chg-Gly-Arg-pNA was approximately the same in both solvents. In the presence of increasing concentrations of the monoclonal Fab, the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA decreased to ∼35−50% of the control value in both sodium chloride and choline chloride. Furthermore, the Fab increased the rate of hydrolysis of MeO-CO-D-Chg-Gly-Arg-pNA to ∼220−260% of the control value in both solvents.

Effect of the Monoclonal IgG on Binding of Hirudin$^{54-65}$ to Exosite I. We used a fluorescent analogue of the C-terminal hirudin dodecapeptide ([5F]Hir$^{54-65}$) to determine if the monoclonal IgG affects the conformation of exosite I in addition to that of the active site. As shown in Figure 7 (open circles), titration of a fixed concentration of [5F]Hir$^{54-65}$ with 0−600 nM thrombin caused a progressive decrease in fluorescence. The data were well described by binding of the peptide to one site on thrombin with a K_D of 23 ± 2 nM, which is in good agreement with previous estimates (*30*). The presence of the monoclonal IgG at saturating concentrations (2 or 4 μM, closed symbols) did not have a significant effect on either the affinity of peptide binding or the magnitude of the fluorescence change (Figure 7 and Table 3).

DISCUSSION

Our results indicate that binding of a monoclonal IgG to thrombin induces an allosteric effect that alters the rate of hydrolysis of synthetic peptide substrates. The epitope for the IgG most likely includes Arg-233, since replacement of this residue by alanine abolishes binding to the IgG (Figure 1). Arg-233 is located near the center of a patch of basic amino acid residues termed anion-binding exosite II (*8*, *11*) and is ∼20 Å away from Ser-195 in the catalytic triad (Figure 8). The data in Figure 2A suggest that binding of the IgG also involves the nearby residues Arg-101 and Lys-236 but not Arg-93, Arg-97, Arg-126, or Lys-169. Other residues that were not examined in the present study (e.g., Arg-165 and Lys-240) may also be involved in binding to the IgG. We have not definitively eliminated the possibility that the R101A, R233A, and K236A mutations cause a conformational change at some distant site to which the IgG binds; nevertheless, we believe that this possibility is unlikely, since these mutations do not affect chromogenic substrate hydrolysis, fibrinogen clotting, protein C activation, thrombomodulin binding, inhibition by an oligonucleotide aptamer, or inhibition by antithrombin in the absence of heparin (*26*, *34*). The only activity, besides binding of the monoclonal IgG, affected by these mutations is heparin-dependent inhibition by antithrombin, consistent with the location of Arg-101, Arg-233, and Lys-236 in the heparin-binding site of thrombin (*34*). Furthermore, the observation that heparin reverses the allosteric effect of the IgG (Figure 2B) is consistent with the hypothesis that heparin and the IgG share a binding site in exosite II.

Our previous finding that the monoclonal IgG binds to both bovine and human thrombin (*24*) is consistent with the

Table 2: Kinetics of Substrate Hydrolysis in the Absence and Presence of Monoclonal IgG[a]

substrate	K_m (μM)		k_{cat} (s^{-1})	
	−IgG	+IgG	−IgG	+IgG
tosyl-Gly-Pro-Arg-pNA	9.5 ± 2.1	2.4 ± 0.5	165 ± 13	89 ± 4
MeO-CO-D-Chg-Gly-Arg-pNA	43 ± 22	30 ± 6	36 ± 6	66 ± 4

[a] The data in Figure 4 were fit to the Michaelis−Menten equation to obtain the K_m and k_{cat} values as described in Experimental Procedures. Error estimates represent ±2 SD.

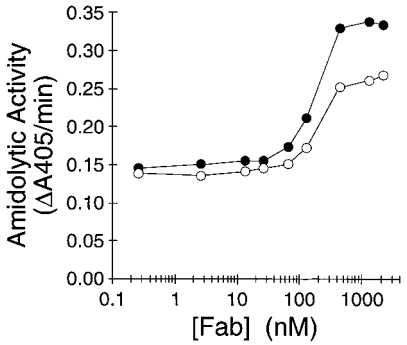

FIGURE 5: Effect of the monoclonal Fab on thrombin(desPPW). Thrombin(desPPW) (55 nM) was preincubated for 1 min at room temperature in 100 μL of TSP buffer containing various concentrations of Fab fragments. Substrate (1000 μM in 500 μL of TSP buffer) was then added, and the absorbance at 405 nm was recorded continuously. The initial rate of hydrolysis of the substrate ($\Delta A405$/min) is shown. Curves: ●, tosyl-Gly-Pro-Arg-pNA; ○, MeO-CO-D-Chg-Gly-Arg-pNA.

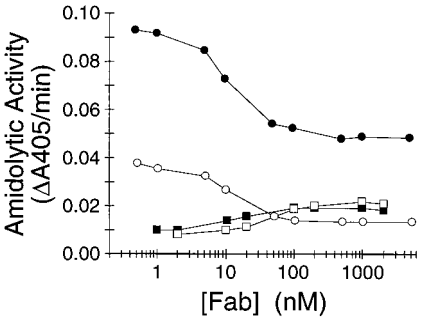

FIGURE 6: Effect of sodium on substrate hydrolysis in the presence of the monoclonal Fab. Thrombin (10 nM) was preincubated with various concentrations of the monoclonal Fab in 100 μL of 5 mM Tris-HCl and 1 mg/mL poly(ethylene glycol) 8000, pH 8.0, containing either 200 mM NaCl (closed symbols) or 200 mM choline chloride (open symbols). Substrate (1000 μM in 500 μL of the same buffer) was then added, and the absorbance at 405 nm was recorded continuously. The initial rate of hydrolysis of the substrate ($\Delta A405$/min) is shown. Curves: ○ and ●, tosyl-Gly-Pro-Arg-pNA; □ and ■, MeO-CO-D-Chg-Gly-Arg-pNA.

fact that Arg-101, Arg-233, and Lys-236 are conserved in both species (*36*). The IgG also recognizes human β- and γ-thrombin (*24*), proteolytic derivatives of α-thrombin with impaired binding of macromolecules to exosite I (*37*). By contrast, the IgG does not recognize human prothrombin (*24*), in which the fragment 2 kringle domain occupies exosite II by forming ion pairs and hydrogen bonds with Arg-93, Arg-97, Arg-101, and Arg-175 (*38*). Similarly, the IgG does not bind to recombinant human meizothrombin or affect its catalytic activity.[3]

Fab fragments of the monoclonal IgG affect the rate at which thrombin cleaves various peptide *p*-nitroanilide substrates with arginine in the P1 position, increasing the k_{cat} for substrates with a P2 glycine residue but generally decreasing the k_{cat} for substrates with a P2 proline (Figure 3 and Table 1). Therefore, binding of the Fab may affect the conformation of the S2 subsite of thrombin. The k_{cat} for at least one substrate with proline in the P2 position (i.e., pyroGlu-Pro-Arg-pNA) increases in the presence of the Fab, however, suggesting that the allosteric effect is more complex and may also involve the S3 subsite and/or the aryl-binding site.

The 60-loop forms a hydrophobic lid that partially covers the S2 subsite and the aryl-binding site of thrombin (*11*). Deletion of part of the 60-loop increases the affinity of thrombin(des PPW) for bovine pancreatic trypsin inhibitor ~3000-fold (*10*), indicating that the loop restricts access of certain macromolecules to the active site. By contrast, the affinity (K_m) of thrombin(desPPW) for the fibrinogen Aα chain is unaffected, whereas the k_{cat} for cleavage of this substrate is decreased ~50-fold (*10*). Therefore, the 60-loop appears to be important for the proper orientation of the scissile bond relative to the catalytic triad. Although the 60-loop has been considered to be a relatively rigid structure, recent crystallographic studies indicate that it can move a

[3] N. S. Colwell and D. M. Tollefsen, unpublished observation.

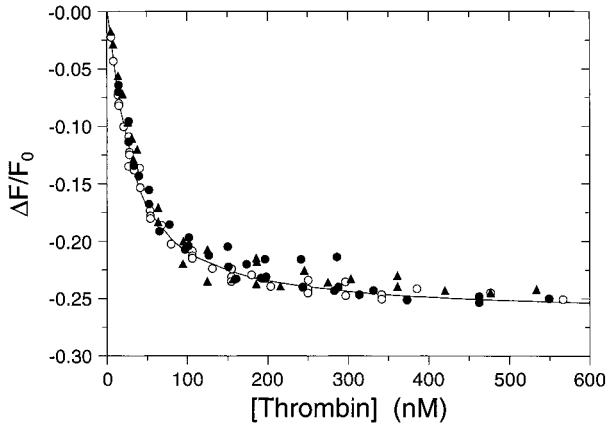

FIGURE 7: Binding of [5F]Hir$^{54-65}$ to thrombin. [5F]Hir$^{54-65}$ was titrated with thrombin as described in Experimental Procedures in the presence of 0 μM (○), 2 μM (●), or 4 μM (▲) monoclonal IgG. The fluorescence change divided by the initial fluorescence ($\Delta F/F_0$) is plotted along with the fitted curve for the data in the absence of IgG with the parameters given in Table 3.

Table 3: Binding of [5F]Hir$^{54-65}$ to Thrombin[a]

IgG (μM)	K_D (nM)	$\Delta F_{max}/F_0$ (%)
0	23 ± 2	−26 ± 1
2	27 ± 4	−26 ± 1
4	29 ± 5	−26 ± 1

[a] The data in Figure 7 were analyzed as described previously (*30*). Error estimates represent ±2 SE. K_D, dissociation constant; $\Delta F_{max}/F_0$, maximal fluorescence change.

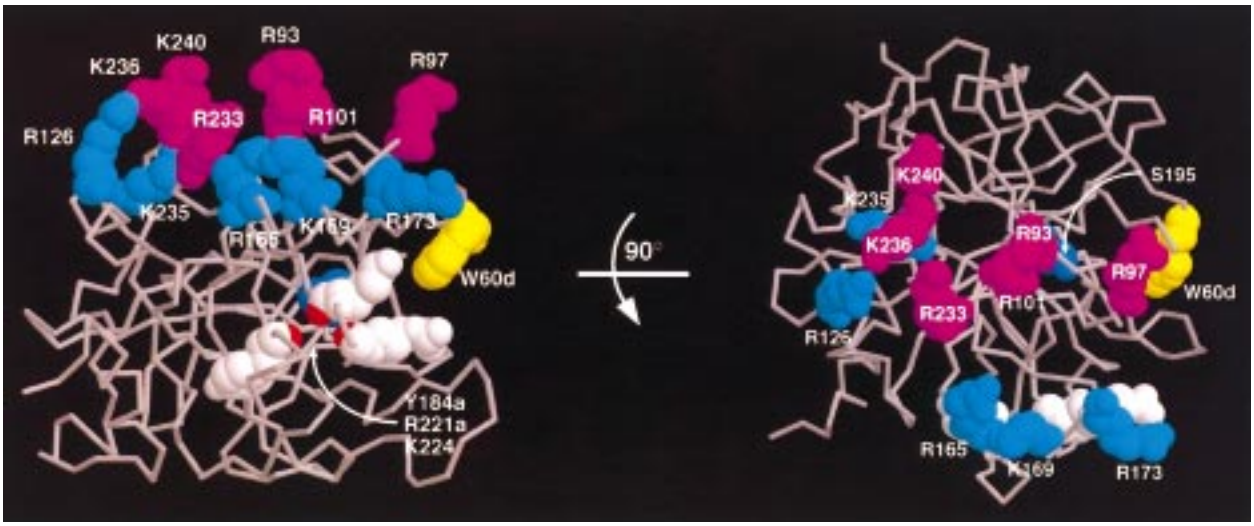

FIGURE 8: Tertiary structure of thrombin. The α-carbon backbone of thrombin is derived from the crystal structure of Bode et al. (*8*). In the left panel, the protein is rotated ~90° to the right about the *y*-axis with respect to the standard view. Arg-233 and other residues in exosite II implicated in binding of glycosaminoglycans or fragment 2 are shown in magenta. Residues in exosite II not implicated in ligand binding (see Discussion) are shown in cyan. Ser-195 in the catalytic site is shown in dark blue and Trp-60d in yellow. The carbonyl oxygen atoms (red) of Tyr-184a, Arg-221a, and Lys-224 (white) comprise the sodium-binding site.

considerable distance (~2−8 Å) when the active site is occupied by a substrate or by an inhibitor (*39*, *40*). Because of its mobility, as well as its proximity to exosite II, the 60-loop could potentially mediate the allosteric effect of the monoclonal Fab. In experiments with thrombin(desPPW) (Figure 5), we observed that the Fab alters the active site conformation of thrombin independent of Pro-60b, Pro-60c, and Trp-60d, although the magnitude and direction (i.e., positive or negative) of the allosteric effect depend on the presence of one or more of these residues in the 60-loop. For example, the rate of hydrolysis of tosyl-Gly-Pro-Arg-pNA by native thrombin decreases by half in the presence of the Fab, while the rate of hydrolysis of the same substrate by thrombin(desPPW) increases approximately 2-fold. Further experiments will be necessary to determine if the Fab induces movement of the 60-loop or of underlying residues in the active site cleft, either of which might explain the observed allosteric effect.

Di Cera and co-workers have reported that thrombin exists in two distinct conformations in equilibrium, termed the "slow" and "fast" forms (*23*). Binding of a sodium ion to the carbonyl oxygen atoms of Tyr-184a, Arg-221a, and Lys-224 (Figure 8) drives the equilibrium in the direction of the fast form. Although originally distinguished by their activity with *p*-nitroanilide substrates (*22*), the fast form has a higher k_{cat}/K_m value for fibrinogen, whereas the slow form has a higher k_{cat}/K_m value for protein C (*41*). To determine if the effects of the monoclonal Fab on thrombin activity are mediated by changes in this equilibrium, we compared the rate of substrate hydrolysis at various Fab concentrations in the presence of sodium or choline ions (Figure 6). In the absence of the Fab, as expected, hydrolysis of tosyl-Gly-Pro-Arg-pNA is slower in buffer containing choline. The Fab decreases the rate of hydrolysis of this substrate to a similar degree in both buffers. Likewise, the Fab increases the rate of hydrolysis of MeO-CO-D-Chg-Gly-Arg-pNA in the presence of either sodium or choline ions. The results of this experiment suggest that sodium has little or no influence on the allosteric effect of the monoclonal Fab with respect to the two substrates tested.

Although prothrombin fragment 2 and glycosaminoglycans are physiologic ligands that bind to exosite II, relatively little is known about the effects of these ligands on the catalytic activity of thrombin. Arg-233 and several other residues (Arg-93, Arg-97, Arg-101, Lys-236, and Lys-240) have been implicated in heparin binding by site-directed mutagenesis or chemical modification of thrombin (*31*−*35*). Some of these residues have also been shown to be involved in binding to dermatan sulfate (*42*) and to the chondroitin sulfate moiety of thrombomodulin (*43*). Other basic residues, which reside mainly at the periphery of exosite II (Arg-126, Lys-165, Lys-169, Arg-173, and Lys-235) (Figure 8), have not been implicated in binding to these ligands. While both heparin (*44*) and chondroitin sulfate (*17*) induce a conformational change in thrombin that can be detected with a fluorescence probe linked to the catalytic site through a tripeptide chloromethyl ketone, attempts to demonstrate an effect of heparin on catalytic activity have been complicated by direct interaction of heparin with the *p*-nitroanilide substrates used (*45*−*47*). Two reports suggested that heparin has no effect on hydrolysis of H-D-Phe-Pip-Arg-pNA (*45*) or H-D-Ile-Pro-Arg-pNA (*48*), in contrast to the results we obtained with the monoclonal Fab using these substrates (Table 1). Prothrombin fragment 2 produces fluorescence changes in probes linked to the catalytic site (*16*), decreases the rate of inhibition of thrombin by antithrombin (*19*), alters the calcium dependence of protein C activation (*21*), and increases the k_{cat} for hydrolysis of tosyl-Arg-*O*-methyl ester (*18*) and tosyl-Gly-Pro-Arg-pNA (*20*) ~2-fold. By contrast, the monoclonal IgG decreases the k_{cat} for hydrolysis of tosyl-Gly-Pro-Arg-pNA (Table 1). Thus, it appears that various ligands which bind to exosite II can have different effects on the conformation of the catalytic site.

Ligands that bind to exosite I, including the C-terminal portion of hirudin (*49*), growth factor-like domains 5 and 6 of thrombomodulin (*49*), the acidic region of the thrombin receptor (*50*), and fibrin (*44*), produce fluorescence changes in probes linked to the catalytic site of thrombin. Some of these ligands also have been shown to alter the kinetics of hydrolysis of peptide *p*-nitroanilide substrates (*49*−*53*).

Interestingly, the C-terminal hirudin peptide increases the k_{cat} of several substrates (H-D-Phe-Pip-Arg-pNA, H-D-Ile-Pro-Arg-pNA, H-D-(γ-Cbo)Lys-Pro-Arg-pNA, and H-D-Hht-Ala-Arg-pNA) whose hydrolysis is inhibited by the monoclonal Fab and decreases the k_{cat} of one substrate (MeO-CO-D-Chg-Gly-Arg-pNA) whose hydrolysis is stimulated by the Fab (*50*). The conformational changes induced in the catalytic site by the hirudin peptide and by the monoclonal Fab, therefore, may be reciprocal in nature. It has been suggested that exosites I and II are allosterically linked, since the affinity of thrombin for the hirudin C-terminal peptide appears to be decreased in the presence of a peptide corresponding to part of prothrombin fragment 2 and vice versa (*54*). We find, however, that the monoclonal IgG does not alter the affinity of thrombin for the hirudin peptide (Figure 7), which suggests that binding of this ligand to exosite II does not cause a significant conformational change in exosite I. Whether heparin or intact prothrombin fragment 2 causes a conformational change in exosite I remains to be determined.

The patient from whom the monoclonal IgG was isolated had a markedly elevated activated partial thromboplastin time (63.9 s; normal range 24.1−32.3 s) but only a minimally elevated prothrombin time (13.4 s; normal range 11.4−13.2 s) (*24*), suggesting a defect in the intrinsic coagulation pathway (*2*). Esmon and Lollar (*55*) have reported that mutation of Arg-93, Arg-97, and Arg-101 in exosite II to alanine greatly interferes with the ability of thrombin to activate factor VIII but appears to affect the rate of activation of factor V to a lesser degree. Since these residues are likely to be blocked when the monoclonal IgG binds to thrombin, the abnormal activated partial thromboplastin time may result from impaired feedback activation of factor VIII by thrombin. The thrombin time and reptilase time of the patient's plasma were also prolonged, suggesting that high concentrations of the IgG (100−400 μM) directly affect fibrin monomer polymerization. When we reduced the IgG concentration to <100 μM by dilution of the patient's plasma with normal plasma, the reptilase time corrected to normal but the thrombin time remained slightly prolonged (18−20 s; normal 13.8 s), suggesting that the IgG also interferes with the ability of thrombin to cleave fibrinogen in plasma (*24*). This effect appears to be mediated by antithrombin, since the thrombin time of antithrombin-deficient plasma is not prolonged by the IgG; moreover, low concentrations of the IgG do not affect clotting of purified fibrinogen but increase the rate of inhibition of thrombin by antithrombin ~3-fold in the absence of heparin.[3]

In summary, we have identified the epitope for a monoclonal IgG that binds to exosite II of thrombin. The IgG affects hydrolysis of peptide *p*-nitroanilide substrates, providing evidence for a direct allosteric linkage between exosite II and the catalytic site of thrombin. We have recently found that either heparin or the IgG induces dissociation of the complex between thrombin and a reactive site variant (Leu-444 → Arg) of the serpin heparin cofactor II (*56*, *57*), presumably by a similar allosteric mechanism. The monoclonal IgG promises to be a useful reagent with which to investigate the interactions of thrombin with other macromolecules.

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