Substitution of Valine for Glycine-558 in the Congenital Dysthrombin Thrombin Quick II Alters Primary Substrate Specificity†

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ABSTRACT: Thrombin Quick II is one of two dysfunctional forms of thrombin derived from the previously described congenital dysprothrombin prothrombin Quick. Thrombin Quick II does not clot fibrinogen, hydrolyze *p*-nitroanilide substrates of thrombin, or bind N^2-[5-(dimethylamino)naphthalene-1-sulfonyl]arginine *N,N*-(3-ethyl-1,5-pentanediyl)amide, a high-affinity competitive inhibitor of thrombin. To determine the structural alteration in thrombin Quick II, the reduced, carboxymethylated protein was hydrolyzed by a lysyl endopeptidase. A peptide not present in a parallel thrombin hydrolysate was identified by reverse-phase chromatography. The peptide was purified by rechromatography and subjected to Edman degradation which showed that Gly-558 of human prothrombin had been replaced by Val. This corresponds to a point mutation of the Gly codon GGC to GUC. This Gly residue, which is highly conserved in the chymotrypsin family of serine proteases, forms part of the substrate binding pocket for bulky aromatic and basic side chains in chymotrypsin and trypsin, respectively. However, in porcine elastase 1, the corresponding residue is threonine. Consistent with the identified structural alteration, thrombin Quick II incorporates [^{3}H]diisopropyl fluorophosphate stoichiometrically and hydrolyzes the elastase substrate succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide with a relative k_{cat}/K_M of 0.14 when compared to thrombin. This results from a 3-fold increase in K_M and a 2.5-fold decrease in k_{cat} for thrombin Quick II when compared to thrombin acting on the same substrate. These results and those of other investigators studying mutant trypsins support the conclusion that the catalytic activity of serine proteases is very sensitive to structural alterations in the primary substrate binding pocket.

Prothrombin Quick, a congenitally dysfunctional form of the blood coagulation factor prothrombin, is isolated from the plasma of an individual with less than 2% of normal prothrombin activity (Quick et al., 1955; Owen et al., 1978). Upon activation, this dysprothrombin gives rise to two dysthrombins, thrombin Quick I and thrombin Quick II (Henriksen et al., 1980; Henriksen & Owen, 1987). Identification of the primary structural defect and functional studies for thrombin Quick I have been described (Henriksen & Mann, 1988; Henriksen & Owen, 1987). In earlier studies of thrombin Quick II, neither binding of the specific thrombin inhibitor N^2-[5-(dimethylamino)naphthalene-1-sulfonyl]arginine *N,N*-(3-ethyl-1,5-pentanediyl)amide nor catalytic activity toward substrates of thrombin was identified (Henriksen & Owen, 1987). Thrombin Quick II has a molecular weight like that of thrombin under both reducing and nonreducing conditions and chromatographic properties on sulfopropyl-Sephadex similar to those of thrombin. The structural studies, reported here, were undertaken to determine the reason for the lack of catalytic activity associated with this protein and possibly to identify a residue essential for the activity of thrombin, a serine protease that has a critical role in the regulation of blood coagulation [for review, see Mann and Lundblad (1987)].

Thrombin has limited trypsin-like specificity such that, in its biologic substrates, hydrolysis occurs following only a limited number of arginine residues. In the thrombin-catalyzed release of fibrinopeptide A from the substrate fibrinogen, specificity determinants include both a secondary substrate binding site, distal to the catalytic site, and a primary binding pocket for arginine, the P_1 residue (Schechter & Berger, 1967), which contributes the carboxyl group to the hydrolyzed bond. Examination of crystallographic structures for serine proteases indicates that the structure of the primary substrate binding pocket is complementary to the P_1 residue. In this study an amino acid substitution in the arginine binding pocket is identified in thrombin Quick II. On the basis of this finding, further predictions regarding properties of the catalytic site have been tested.

Experimental Procedures

Reagents. [^{3}H]Diisopropyl fluorophosphate ([^{3}H]DFP)[1] with a specific activity of 4.4 Ci/mmol was obtained from New England Nuclear, Boston, MA. Scintillation fluid was Beckman Ready-Solve EP. Sephadex G-25, a product of Pharmacia Fine Chemicals, Inc., DFP, and succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide were obtained from Sigma Chemical Co., St. Louis, MO. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone was purchased from Calbiochem, San Diego, CA. Other reagents were as described (Henriksen & Mann, 1988).

Thrombin Preparations. Thrombin Quick II was prepared as described previously (Henriksen & Owen, 1987) from prothrombin Quick that was isolated from plasma obtained by pheresis from the donor V.A. (Henriksen et al., 1980; Quick et al., 1955; Quick, 1974). Approval of the University of Iowa Human Subjects Committee (Iowa City, IA) and informed consent of the donor were obtained. The thrombin Quick II

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; PTH, phenylthiohydantoin; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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preparation contained about 1% thrombin Quick I as determined by hydrolysis of Tos-Gly-Pro-Arg-*p*-nitroanilide. In experiments measuring the hydrolytic activity of thrombin Quick II, the covalent inhibitor of thrombin D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone was added to inhibit residual thrombin Quick I. Human α-thrombin was prepared for structural studies as described (Lundblad et al., 1976) or for studies of the active-site serine as described (Henriksen & Owen, 1987). Concentrations of thrombin and thrombin Quick II were determined from the absorbance at 280 nm after correction for light scattering at 320 nm by using $E = 1.74$ mL mg^{-1} cm^{-1} (Fenton et al., 1977) and $M_r = 36000$.

Peptides. Peptides of thrombin and thrombin Quick II were prepared by lysyl endopeptidase digestion and isolated by reverse-phase chromatography on a Vydac 218TP54 C-18 column (The Sep/a/ra/tions Group, Hesperia, CA) as described for thrombin Quick I (Henriksen & Mann, 1988). In addition, peptide maps were prepared by chromatography on an RP-300 (C-8) cartridge, 3 cm × 4.6 mm (Brownlee Labs, Santa Clara, CA), with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. This column effluent was monitored by fluorescence following postcolumn derivatization with *o*-phthalaldehyde (Benson & Hare, 1975; Henriksen & Mann, 1988). Peptides were sequenced by automated Edman degradation with the Applied Biosystems, Inc. (Foster City, CA), Model 470A gas-phase sequencer connected to the Applied Biosystems Model 120A analyzer for on-line identification of PTH-amino acids (Hunkapiller et al., 1983). The PTH-amino acids were quantitated by comparing peak heights to those of a 25-pmol standard injected prior to sequencing each peptide. To quantitate Thr, His, and Arg, results from freshly reconstituted standards were used after normalizing to the results from each peptide run by comparing the peak heights for Phe, Val, Pro, Met, Ile, and Gly.

[^{3}H]DFP Incorporation. [^{3}H]DFP was added to 20 mM unlabeled DFP in dimethylformamide to give a final concentration of 17 mM DFP, with a specific activity of 10 Ci/mol. The [^{3}H]DFP solution (0.05 mL) to a final concentration of 1.5 mM was added to 0.50 mL of thrombin, thrombin Quick II, or bovine serum albumin, each at 0.5 mg/mL, and incubated at 22 °C for 2–4 h. Bovine serum albumin was used as a control for nonspecific binding of DFP. Each protein was desalted on a 0.6 × 15 cm column of Sephadex G-25. Fractions were collected and the protein concentration was determined from the absorbance at 280 nm. Samples from protein-containing fractions were subjected to liquid scintillation counting to determine the amount of DFP incorporated into each protein. Counts per minute (cpm) were converted to cpm/mg of protein, and the resultant activities for thrombin and thrombin Quick II were corrected for nonspecific binding to albumin. The radioactivity associated with albumin was 7–11% of the total radioactivity found in thrombin or thrombin Quick II.

Hydrolysis of Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. Hydrolysis of this substrate by thrombin and thrombin Quick II was studied at 30 °C, in 0.1 m NaCl–0.05 M Tris, pH 8.3, by following the change in absorbance at 405 nm, on a Cary 219 spectrophotometer. Substrate concentrations reported here were determined by weight. From the absorbance at 342 nm and assumed $\epsilon = 8270$ (Lottenberg & Jackson, 1983), the substrate concentration was 0.89 that determined by weight. The rate of hydrolysis was determined by using $\epsilon = 10^4$ for *p*-nitroaniline (Svendsen & Stocker, 1977). For thrombin, the substrate concentration was varied from 0.2 to 7 times K_M, and for thrombin Quick II, the concentration varied from 0.2 to 2.3 times K_M. Except where indicated, the final concentrations of thrombin and thrombin Quick II were 0.31 and 0.63 μM, respectively. Initial rate data were analyzed and fitted with the program HYPER as described (Henriksen & Owen, 1987; Cleland, 1979).

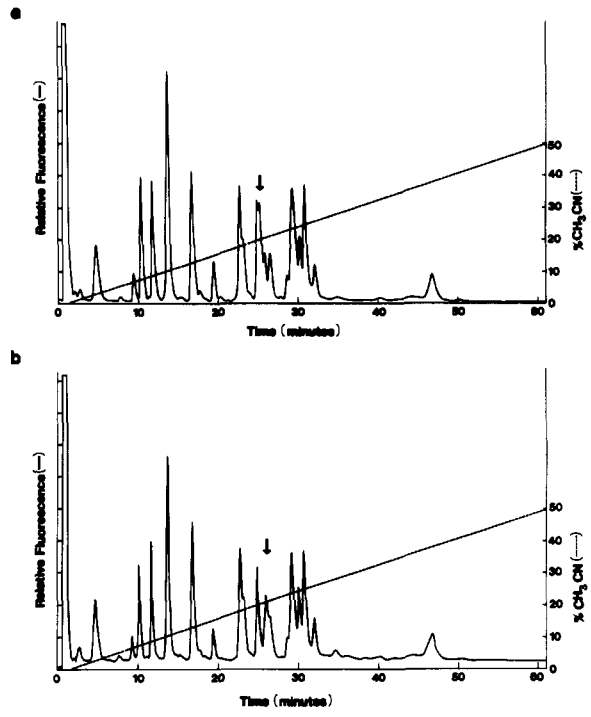

FIGURE 1: Peptide maps for thrombin and thrombin Quick II. Reverse-phase chromatography on an RP-300 cartridge column for peptides derived from a lysyl endopeptidase digest of S-carboxymethylated thrombin (a) or thrombin Quick II (b). Chromatograms show relative fluorescence following postcolumn derivatization with *o*-phthalaldehyde. The altered elution position for the mutant peptide is indicated by the vertical arrows.

Molecular Modeling. The molecular graphics software package PROMODELER from New England Biographics, Peachem, VT, was used to investigate the effects of amino acid substitution within the tertiary structure of thrombin. The coordinates for the thrombin model were those determined previously (Furie et al., 1982). Pictures were obtained by directly photographing the monitor screen in a darkened room.

RESULTS

Peptide Isolation. The results of peptide mapping studies on the RP-300 column are shown in Figure 1. A peptide missing in thrombin Quick II is indicated in the chromatogram for thrombin as is an additional peptide present in thrombin Quick II. These results were used to identify the fractions containing the altered peptide in the preparative scale chromatogram. To isolate peptides for sequencing, material from both thrombin and thrombin Quick II that was eluted from the Vydac 218TP54 C-18 column in the regions of the altered peptides was concentrated by lyophilization and subjected to rechromatography, at pH 6.5, on the same column (Miyata et al., 1987; Henriksen & Mann, 1988). Comparison of chromatograms for the two sets of peptides indicated the unique peptides in each protein.

Identification of the Substitution of Val for Gly-558. The peptide from thrombin that was missing in thrombin Quick II was subjected to automated Edman degradation, as was the peptide unique to thrombin Quick II. These results, shown in Table I with recoveries and repetitive yields of PTH-amino acids, indicate that the altered residue in thrombin Quick II is Gly-558[2] of the human prothrombin sequence (Butkowski

Table I: Identification of Altered Amino Acid in Thrombin Quick II[a]

cycle	prothrombin sequence position	thrombin			thrombin Quick II				
		amino acid identified	pmol	repetitive yield	amino acid identified	pmol	repetitive yield	pmol	repetitive yield
1	557	Tyr	63		Tyr	31		128	
2	558	Gly	50		Val	26		119	
3	559	Phe	61		Phe	30		114	
4	560	Tyr	56	0.96	Tyr	28	0.97	123	0.99
5	561	Thr	16		Thr	7		30	
6	562	His	13		His	7		NI[b]	
7	563	Val	27		Val	12	0.86	52	0.86
8	564	Phe	29	0.86	Phe	14	0.86	52	0.86
9	565	Arg	12		Arg	6		26	
10	566	Leu	18		Leu	8		26	
11	567	Lys	11		Lys	4		16	
12		none			none				

[a] Results given for thrombin Quick II are duplicate determinations for the same peptide preparation. Sequence position numbers are according to Degen et al. (1983). [b] Residue in this cycle was not identified.

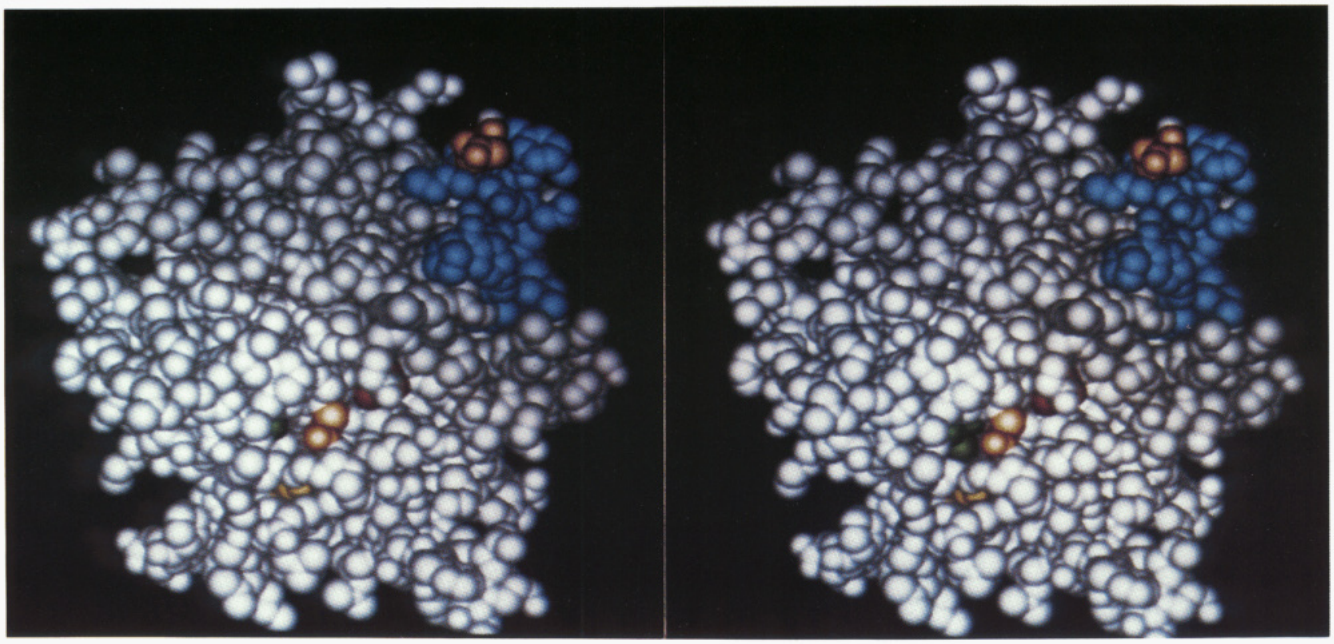

FIGURE 2: Space-filling models of thrombin and thrombin Quick II. The model on the left is for thrombin with the two glycine residues forming part of the arginine binding pocket shown in green and orange in the lower center (residues 226 and 216, respectively, in chymotrypsin). The model on the right is for thrombin Quick II where the corresponding green residue shows the effect of substituting valine at position 558 (residue 226 in chymotrypsin). Other residues shown in color are Ser-195 in red and Asp-189 at the base of the Arg binding pocket in yellow. The blue residues at the upper right of the model form the B insertion loop with the Asn carrying the carbohydrate side chain shown in orange (Elion et al., 1977). The thrombin model is that developed by Furie et al. (1982).

et al., 1977; Degen et al., 1983), which is replaced by Val. No alternate sequences were detected in sequencing either the normal or abnormal peptide.

Residue 558 in human prothrombin corresponds to glycine-226 in the trypsin and chymotrypsin sequences where it is part of the primary substrate binding pocket. To better understand the possible effects of this substitution on the structure of thrombin, the thrombin model previously described (Furie et al., 1982) was used. The effect of substituting Val for Gly at position 558 (residue 226 in chymotrypsin) is shown by the space-filling models in Figure 2.

Stoichiometric Incorporation of DFP by Thrombin Quick II. Although previous work did not identify catalytic activity associated with thrombin Quick II, the primary structural studies described above indicated that the catalytic triad residues common to serine proteases should be present in thrombin Quick II. Therefore, the incorporation of [^{3}H]DFP into thrombin and thrombin Quick II was investigated in parallel studies to verify the presence of the active-site serine. The results from two separate experiments were averaged and showed the incorporation of 1.58×10^5 cpm mg^{-1} into thrombin and 1.62×10^5 cpm mg^{-1} into thrombin Quick II, yielding a ratio of 1.03 for thrombin Quick II relative to thrombin. As predicted by the primary structural findings, these results are consistent with the presence of an active-site serine in thrombin Quick II.

Kinetics of Hydrolysis of Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. Gly-226 is conserved in serine proteases of the chymotrypsin family except in the case of elastase, which most efficiently hydrolyzes both peptides and *p*-nitroanilides following small nonpolar amino acid residues (Narayanan & Anwar, 1969; Kasafirek et al., 1976). This observation and the stoichiometric incorporation of DFP suggested that hydrolytic activity might be observed for thrombin Quick II on an elastase substrate. Several biologic substrates for thrombin

[2] Sequence numbers for thrombin refer to the human prothrombin sequence. For other serine proteases, the positions in the primary structure are designated according to the primary structure of chymotrypsinogen.

Table II: Comparison of Kinetic Constants for Hydrolysis of Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide

enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	conditions
thrombin[a]	2.0 ± 0.3	0.079 ± 0.004	41 ± 4	30 °C, 0.1 M NaCl, 0.05 M Tris·HCl, pH 8.3
thrombin Quick II[a]	5.6 ± 1.5	0.032 ± 0.004	5.7 ± 0.9	30 °C, 0.1 M NaCl, 0.05 M Tris·HCl, pH 8.3
human pancreatic elastase 2[b]	1.4	5.1	3600	25 °C, 0.2 M Tris·HCl, pH 8.0
porcine elastase 1[b]	1.2	18	14000	25 °C, 0.2 M Tris·HCl, pH 8.0

[a] Results are ±SE. [b] Results taken from Del Mar et al. (1980).

contain proline in the P_2 position (Schechter & Berger, 1967) as does the synthetic substrate for thrombin, Tos-Gly-Pro-Arg-*p*-nitroanilide. Therefore, the substrate succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was selected for comparison of steady-state kinetic constants between thrombin and thrombin Quick II. As predicted from the structure of thrombin Quick II, this substrate is hydrolyzed, although less efficiently than by thrombin with thrombin Quick II having both an increased K_M and a decreased k_{cat} relative to thrombin. The results obtained by fitting the kinetic data to the hyperbolic form of the Michaelis–Menten equation are shown in Table II. Activity is linear in enzyme concentration for thrombin from 0.06 to 0.6 μM and for thrombin Quick II from 0.2 to 0.9 μM. For comparison, the kinetic constants obtained by other investigators for two elastases acting on this substrate are also given in Table II.

Discussion

The primary structural studies presented above have identified Gly-558 in the human prothrombin sequence as the site of substitution in thrombin Quick II. The substitution by valine of this residue within the primary substrate binding site is expected to limit access of arginine residues (see Figure 2) and provides an explanation for results obtained in earlier studies where catalytic activity was not observed for this dysthrombin. The identical behavior of thrombin and thrombin Quick II, observed previosuly, on isoelectric focusing gels is consistent with the substitution of a residue by one of like charge (Henriksen, 1985). The amino acid composition of thrombin Quick II (Henriksen, 1985) and the identical chromatographic behavior of the other peptides obtained by lysyl endopeptidase hydrolysis of thrombin and thrombin Quick II are consistent with the absence of other alterations in the primary structure of thrombin Quick II.

The substitution of Val for Gly in thrombin Quick II is consistent with a transversional point mutation in a prothrombin gene of the affected individual. This mutation would result in the conversion of the codon GGC, for Gly-558, to GUC. Gly to Val mutations, although only occasionally reported, have been identified previously at positions 24 and 74 in the hemoglobin β-chain (Huisman et al., 1971; Rieder et al., 1975).

The earlier report demonstrating the substitution of Cys for Arg-382 in thrombin Quick I (Henriksen & Mann, 1988), and the finding of a different amino acid substitution in thrombin Quick II, confirms the earlier conclusion, based on the functional characterization of these two proteins, that the affected individual is heterozygous for two dysfunctional prothrombin molecules (Henriksen & Owen, 1987). This work represents the completion of the first primary structural characterization of two dysprothrombins isolated from the plasma of one individual. Because the gene for prothrombin is autosomal, in the absence of consanguinity, finding two gene products in cases of severe functional prothrombin deficiency should not be unanticipated. It may be noted that characterization of the dysprothrombinemia in this individual by DNA sequencing of a clone from a single gene might easily have overlooked the second mutant gene.

In serine proteases, the primary substrate specificity is determined by the structure of the binding pocket for the side chain of the P_1 residue. For those enzymes with trypsin-like specificity, Asp-189 at the base of the P_1 binding pocket is thought to play a significant role in binding the side chains of arginine or lysine. By comparison of the crystal structures for trypsin and porcine elastase, two additional residues, at positions 216 and 226, have been identified as being critical in determining specificity at the P_1 site. These two residues, which form part of the P_1 binding pocket and are highly conserved as glycine in the chymotrypsin family of serine proteases, are thought to be important for allowing access of larger amino acid side chains. In elastase, positions 216 and 226 correspond to valine and threonine, respectively. These bulkier side chains sterically block the binding of larger side chains such as those of arginine or the aromatic amino acids to the P_1 binding pocket (Shotton & Watson, 1970).

For the region including residues 216–226, there are eight substitutions and two insertions in the elastase sequence when aligned with human or bovine prothrombin (Shotton & Hartley, 1973; Degen et al., 1983; MacGillivray & Davie, 1984). This suggests that differences in catalytic specificity between thrombin and elastase are more extensive than the simple substitution of two bulky residues into the arginine (P_1) binding site of thrombin, consistent with the lower affinity toward the elastase substrate succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide when thrombin or thrombin Quick II is compared to elastase (Table II). Previous work found that thrombin has limited hydrolytic activity toward the *p*-nitrophenyl ester of acetylleucine (Lorand et al., 1962), in agreement with the observation presented here that a leucyl-*p*-nitroanilide bond is hydrolyzed by thrombin. For thrombin Quick II acting on the elastase substrate succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, results in Table II, the relative activity is 0.14 of that for thrombin when results for k_{cat}/K_M are compared. This is the result of a 3-fold increase in K_M and a 2.5-fold decrease in k_{cat}. The increase in K_M for thrombin Quick II suggests that the substitution of glycine in thrombin by a valine residue not only prevents binding of Arg at the P_1 site but also interferes with the binding of the somewhat smaller Leu residue.

Through site-specific mutagenesis of trypsin, alanine has been substituted for Gly-216 and Gly-226. When either one or both of these residues were substituted, the resulting product displayed a decrease in k_{cat}/K_M ranging from 1/60 to $1/(3 \times 10^4)$ of that for the wild-type enzyme when substrates with Arg or Lys were used in the P_1 position. Although substrates with other amino acid residues in the P_1 position were not studied, the results indicate that the P_1 binding site is very sensitive to structural alterations (Craik et al., 1985). Two additional mutant serine proteases in which the fundamental defect appears to involve the relationship of the catalytic triad residues have been described. These include the plasmin derived from plasminogen Tochigi in which Ala-55[2] is replaced by Thr (Miyata et al., 1982). This Ala is highly conserved in serine proteases of the chymotrypsin family and is two residues removed in the sequence from His of the catalytic triad. The other case is thrombin Tokushima in which Arg-418 in the human prothrombin sequence is replaced by Trp, re-

sulting in decreased activity toward both synthetic and biological substrates (Miyata et al., 1987). Although this arginine is not a conserved residue, it is located adjacent to Asp of the catalytic triad residues.

The results presented here together with the previously reported functional properties of thrombin Quick II (Henriksen & Owen, 1987) establish that residue 558 in human prothrombin is critical for controlling the primary substrate specificity in thrombin and support the findings of other investigators who have identified the essential role of this conserved glycine residue in the primary substrate binding pocket of serine proteases.

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Registry No. Gly, 56-40-6; Val, 72-18-4; thrombin Quick II, 117183-23-0; thrombin, 9002-04-4; succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, 70968-04-6.

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