Gryczynski, I., Wiczk, W., Johnson, M. L., Cheung, H. C., Wang, C. K., & Lakowicz, J. R. (1988b) *Biophys. J.* (submitted for publication).
Haas, E., Wilchek, M., Katchalski-Katzir, E., & Steinberg, I. Z. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1807-1811.
Haas, E., Katchalski-Katzir, E., & Steinberg, I. Z. (1978) *Biochemistry 17*, 5064-5070.
Johnson, M. L. (1983) *Biophys. J. 44*, 101-106.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Chapter 9, pp 257-301, Plenum, New York.
Lakowicz, J. R., & Cherek, H. (1980) *J. Biol. Chem. 255*, 831-834.
Lakowicz, J. R., & Weber, G. (1980) *Biophys. J. 32*, 591-601.
Lakowicz, J. R., & Maliwal, B. P. (1985) *Biophys. Chem. 21*, 61-78.
Lakowicz, J. R., Laczko, G., Cherek, H., Gratton, E., & Limkeman, M. (1984) *Biophys. J. 46*, 463-477.
Lakowicz, J. R., Cherek, H., Maliwal, B. P., & Gratton, E. (1985) *Biochemistry 24*, 376-383.
Lakowicz, J. R., Laczko, G., & Gryczynski, I. (1986a) *Rev. Sci. Instrum. 57*, 2499-2506.
Lakowicz, J. R., Laczko, G., Gryczynski, I., & Cherek, H. (1986b) *J. Biol. Chem. 261*, 2240-2245.
Lakowicz, J. R., Laczko, G., & Gryczynski, I. (1987a) *Biochemistry 26*, 82-90.
Lakowicz, J. R., Johnson, M. L., Gryczynski, I., Joshi, N., & Laczko, G. (1987b) *J. Phys. Chem. 91*, 3277-3285.
Lakowicz, J. R., Cherek, H., Gryczynski, I., Joshi, N., & Johnson, M. L. (1987c) *Biophys. Chem. 28*, 35-50.
Lakowicz, J. R., Joshi, N. B., Johnson, M. J., Szmacinski, H., & Gryczynski, I. (1987d) *J. Biol. Chem. 262*, 10907-10910.
Maliwal, B. P., & Lakowicz, J. R. (1986) *Biochim. Biophys. Acta 873*, 161-172.
McWerther, C. A., Haas, E., Lied, A. R., & Scheraga, H. A. (1986) *Biochemistry 25*, 1951-1963.
Murray, A. C., & Kay, C. M. (1972) *Biochemistry 11*, 2622-2627.
O'Connor, D. V., & Phillips, D. (1985) *Time-Correlated Single Photon Counting*, Academic, New York.
Perry, S. V., & Cole, H. A. (1974) *Biochem. J. 141*, 733-743.
Premilat, S., & Herman, J., Jr. (1973) *J. Chem. Phys. 59*, 2602-2612.
Premilat, S., & Margret, B. (1979) *J. Chem. Phys. 66*, 3418-3425.
Steinberg, I. Z. (1971) *Annu. Rev. Biochem. 40*, 83-114.
Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819-846.
Visser, A. J. W. G., & Wampler, J. R. (1985) *Anal. Instrum. (N.Y.) 14*, 193-566.
Wang, C.-K. (1985) Ph.D. Thesis, University of Alabama at Birmingham.
Wang, C.-K., & Cheung, H. C. (1986) *J. Mol. Biol. 191*, 509-521.
Wilkinson, J. M. (1974) *Biochim. Biophys. Acta 359*, 379-388.

Identification of the Primary Structural Defect in the Dysthrombin Thrombin Quick I: Substitution of Cysteine for Arginine-382†

Ruth Ann Henriksen*,‡ and Kenneth G. Mann
Department of Biochemistry, The University of Vermont, Burlington, Vermont 05405
Received April 26, 1988; Revised Manuscript Received July 29, 1988

ABSTRACT: A congenitally dysfunctional form of prothrombin, prothrombin Quick, was isolated from the plasma of an individual with less than 2% of normal prothrombin activity. Following activation of prothrombin Quick, two dysfunctional thrombins, thrombin Quick I and thrombin Quick II, were isolated. Functional characterization of thrombin Quick I indicated an increase in K_M and a decrease in k_{cat}, relative to thrombin, for release of fibrinopeptide A. Comparison of k_{cat}/K_M for thrombin Quick I to the value obtained for thrombin yielded a relative catalytic efficiency of 0.012 for thrombin Quick I [Henriksen, R. A., & Owen, W. G. (1987) *J. Biol. Chem. 262*, 4664-4669]. Lysyl endopeptidase digestion of reduced and S-carboxymethylated thrombin and thrombin Quick I has resulted in the identification of an altered peptide in this dysthrombin. Edman degradation of the isolated peptide has shown that the altered residue in this protein is Arg-382 which is replaced by Cys. This could result from a point mutation in the Arg codon, CGC, to yield TGC. Together, these results indicate that Arg-382 is a critical residue in determining the specificity of thrombin toward fibrinogen. Similar relative activities for thrombin Quick I in stimulating platelet aggregation, in the release of prostacyclin from human umbilical vein endothelium, and in the release of fibrinopeptide A suggest that these activities of thrombin share the same specificity determinants.

Thrombin, a serine protease with limited substrate specificity, is derived from the plasma zymogen prothrombin. Thrombin participates directly as a procoagulant in conversion of fibrinogen to fibrin in the final stages of blood coagulation and also has additional regulatory activities that both enhance and attenuate the hemostatic response [for a review, see Mann and Lundblad (1987)]. The identification of specificity determinants in the interaction of thrombin with substrates, inhibitors, and cofactors contributes to the development of models for structure–function relationships in this 36 000 molecular weight monomeric enzyme. Approaches to understanding structure–function relationships in this enzyme have included the study of degraded and chemically modified

† This work was supported by NIH Grants HL 17430 (R.A.H.) and HL 34575 (K.G.M.) and by a grant from the American Heart Association, Iowa Affiliate.
* Address correspondence to this author. Recipient of NSF Visting Professorship for Women Award 1987-88, Grant RII-8700669.
‡ Present address: Division of Allergy, Department of Medicine, East Carolina University School of Medicine, Greenville, NC 27858-4354.

0006-2960/88/0427-9160$01.50/0

forms of thrombin. The interpretation of the results of these studies has been limited by the extensive modification and often incomplete characterization of the degraded forms of thrombin and the lack of specific, stoichiometric substitution at identified sites in the primary structure for chemically modified thrombins.

Dysprothrombinemia is a rarely described congenital abnormality of prothrombin, and even less frequently does a dysprothrombin yield a dysthrombin upon activation of the zymogen. The dysprothrombin prothrombin Quick was isolated from the plasma of an individual with less than 2% of normal prothrombin activity. Following activation of prothrombin Quick, two dysfunctional thrombins, thrombin Quick I and thrombin Quick II, were isolated. Thrombin Quick I has nearly normal activity in hydrolyzing low molecular weight substrates and is indistinguishable from normal thrombin in binding the specific inhibitor, N^2-[5-(dimethylamino)-naphthalene-1-sulfonyl]arginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide. However, in the release of fibrinopeptide A from fibrinogen, there is an increase in K_M from 7 μM for normal thrombin to 44 μM for thrombin Quick I, and a corresponding decrease in k_{cat} from 91 to 7 s^{-1}. The ratio of k_{cat}/K_M for thrombin Quick I compared to thrombin is 0.012 (Henriksen & Owen, 1987). The primary structural studies on thrombin Quick I which are reported here have been undertaken to identify a site in thrombin that is essential in determining specificity toward fibrinogen.

Experimental Procedures

Reagents. Lysyl endopeptidase from *Achromobacter lyticus* was obtained from Wako Chemicals, Inc., Dallas, TX. The lyophilized enzyme was dissolved in sterile 0.15 M NaCl/0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl),[1] pH 7.4, at a concentration of 10 activity units/mL and stored at 4 °C. The activity units (AU) of the enzyme are those defined by the manufacturer. Iodoacetic acid was purchased from Sigma Chemical Co., St. Louis, MO, and was recrystallized from *n*-heptane. Brij 35 as a 30% solution was obtained from Pierce Chemical Co., Rockford, IL, and *o*-phthalaldehyde was obtained from Serva Fine Biochemicals, Inc., Westbury, NY. PTH-(carboxymethyl)cysteine and PTH-Gln were obtained from Sigma Chemical Co. and Pierce Chemical Co. Water and acetonitrile for peptide isolation and purification were HPLC grade.

Peptide Preparation. Thrombin Quick I was prepared as described (Henriksen & Brotherton, 1983) 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. Normal human thrombin was prepared as described (Lundblad et al., 1976). For reduction and carboxymethylation, approximately 1 mg of protein was dialyzed against distilled water. The precipitated protein was lyophilized and dissolved in 0.5 mL of 6 M guanidine hydrochloride, 0.02% disodium ethylenediaminetetraacetic acid, and 0.2 M Tris·HCl, pH 8.6. Mercaptoethanol was added to 1% final concentration, and the mixture was incubated at 37 °C for 2 h prior to the addition of an equimolar amount of iodoacetic acid and incubation at 23 °C for 30 min (Butkowski et al., 1977). The modified protein was transferred to Spectra/Por 6 dialysis tubing, molecular weight cutoff 2000 (Spectrum Medical Industries, Inc., Los Angeles, CA), and dialyzed against distilled water. The precipitated carboxymethylated polypeptide chains were lyophilized and digested with 0.05 AU of lysyl endopeptidase at 37 °C for 4 h as described (Miyata et al., 1987a), in a final volume of 0.50 mL, followed by a second addition of 0.05 AU of lysyl endopeptidase and incubation for 4 additional h. The digested material was stored at −70 °C.

Peptide Isolation. Peptide maps were prepared from 10 μg of the digested protein by reverse-phase chromatography on a Vydac 218TP54 C-18 column (The Sep/a/ra/tions Group, Hesparia, CA) with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. The column effluent was monitored at 214 nm and by fluorescence following postcolumn derivatization with *o*-phthalaldehyde reagent (Benson & Hare, 1975) added to the column effluent at 0.2 mL/min. The *o*-phthalaldehyde reagent contained 1.0 g of *o*-phthalaldehyde, 1.5 mL of β-mercaptoethanol, and 1.5 mL of 30% Brij 35 in 1.0 L of 0.5 M potassium borate buffer, pH 10.4. A Varian Fluorichrom fluorometer was used to monitor fluorescence. Peptides for sequencing were isolated from 200 μg of protein by using the same column and gradient conditions. Peptides were identified by the absorbance at 214 nm, and fractions were collected at 1-min intervals. The fractionated material was stored at −20 °C. Fractions containing the peptides of interest, as determined from the peptide maps, were pooled and concentrated by lyophilization. This material was rechromatographed on the same column by using a gradient of 0–50% acetonitrile into 10 mM ammonium formate, pH 6.5, for 60 min at a flow rate of 1.0 mL/min (Miyata et al., 1987a). Fractions were collected at 1.0-min intervals. Equipment for HPLC consisted of two Beckman Model 112 pumps with a Beckman Model 421 gradient controller and a Waters Model 481 variable-wavelength detector. All peptide chromatography was performed at 22 °C, without temperature regulation. The fractionated material was either used directly or concentrated by lyophilization prior to sequencing.

Peptide Sequencing. Amino acid sequence analysis was performed with an Applied Biosystems Inc. (Foster City, CA) Model 470A gas-phase sequencer connected to an Applied Biosystems Model 120A analyzer (Hunkapiller et al., 1983) for on-line chromatographic analysis of the PTH-amino acids. To identify amino acids and calculate recoveries, PTH-amino acid standards were chromatographed prior to sequencing each peptide. To quantitate Asp, Asn, Thr, Glu, and Arg, results from freshly reconstituted standards were used after normalizing the results to those from each peptide run by comparing the peak heights for Phe, Val, Pro, Met, Ile, and Gly.

Identification of PTH-Gln and PTH-(carboxymethyl)cysteine. Because the PTH derivatives of (carboxymethyl)cysteine and glutamine coelute in the chromatographic system that was used to separate the PTH-amino acids, these two amino acids were identified by resequencing the peptide known to contain either Gln or (carboxymethyl)cysteine with adjustment of the initial chromatography buffer to pH 4.5. This resulted in the separation of the two PTH derivatives with PTH-(carboxymethyl)cysteine eluting near Asn.

Results

Peptide Maps. The results of peptide mapping studies are shown in Figure 1. This analytical-scale chromatography indicated the loss of peptide material for thrombin Quick I in the region labeled A and the appearance of a shoulder in the region labeled B.

[1] Abbreviations: AU, activity unit(s); PTH, phenylthiohydantoin; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography.

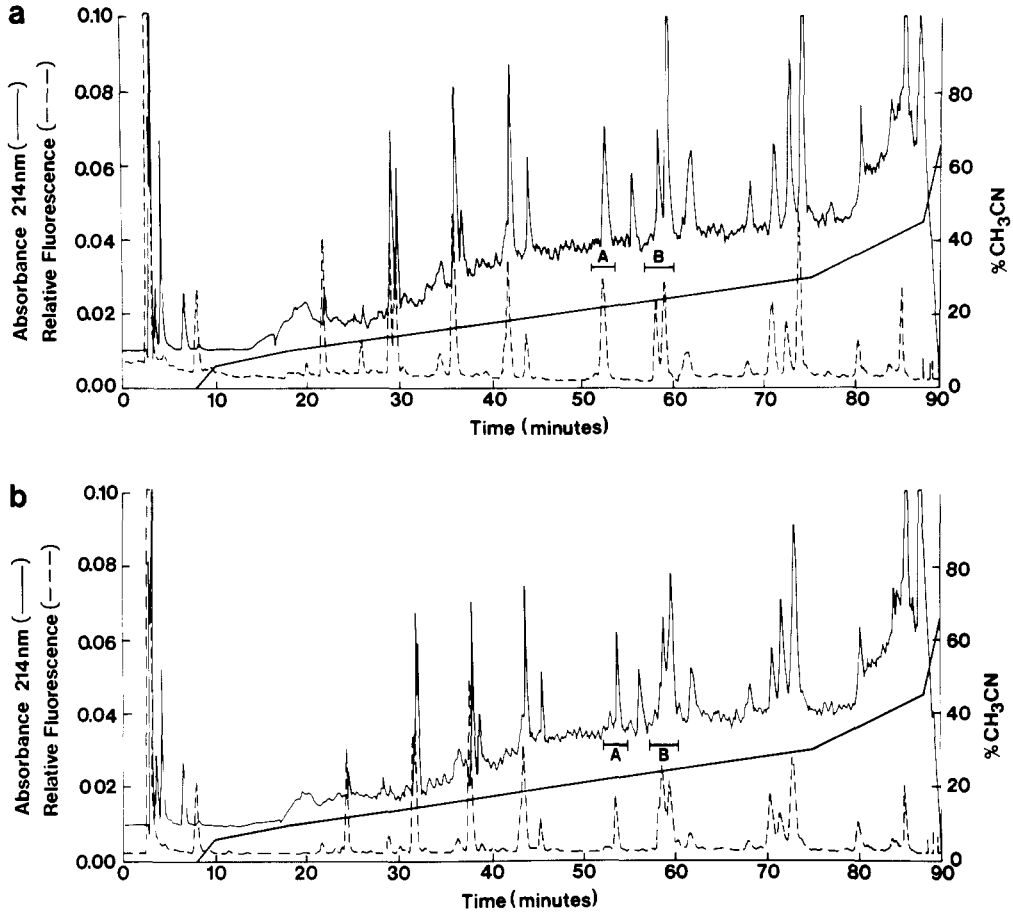

FIGURE 1: Peptide maps for thrombin and thrombin Quick I. Reverse-phase chromatography of peptides derived from a lysyl endopeptidase digest of S-carboxymethylated thrombin (a) or thrombin Quick I (b). Peptide material is missing from thrombin Quick I in the region labeled A, and an additional shoulder appears in the region labeled B.

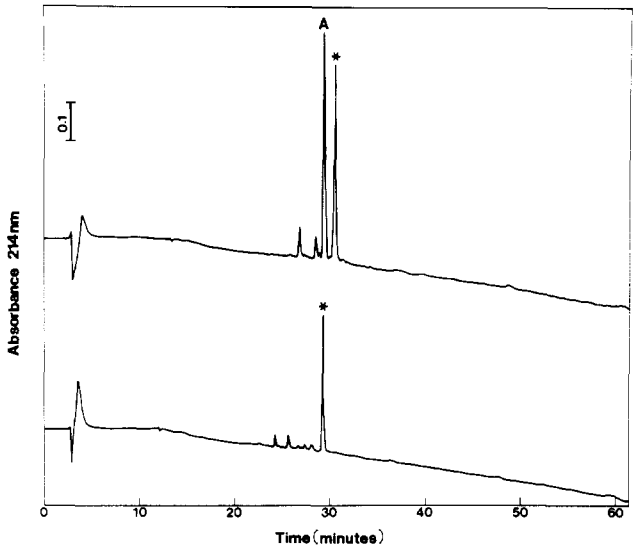

FIGURE 2: Preparative-scale purification of peptides from region A. Peptides from 200 μg of the lysyl endopeptidase digest were chromatographed as in Figure 1. Material from region A was concentrated and rechromatographed at pH 6.5, yielding the chromatograms shown here. The upper tracing is for thrombin, and the lower tracing, demonstrating the absence of one peptide, is for thrombin Quick I. The amino acid sequence obtained for a sample from the peak labeled A in the thrombin chromatogram is given in Table I. The amino-terminal sequence Asp-Ser-X-Arg-Ile-Arg was identified for both peptides labeled with an asterisk, corresponding to the peptide beginning at prothrombin residue 495.

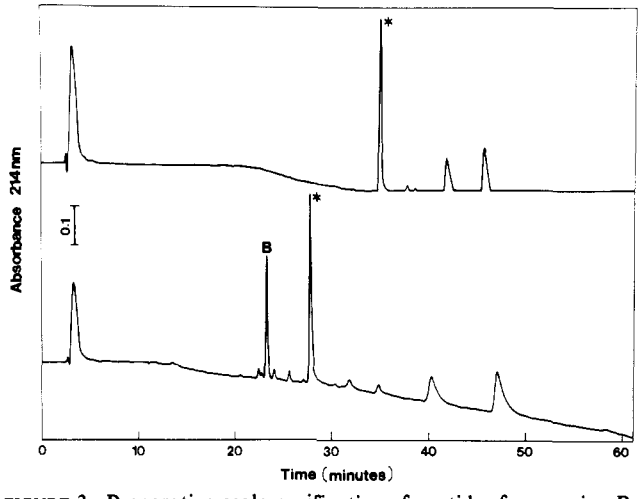

FIGURE 3: Preparative-scale purification of peptides from region B. Peptides from region B were isolated and rechromatographed as described for Figure 2. The upper chromatogram is for thrombin and the lower chromatogram, demonstrating the appearance of an additional peptide, is for thrombin Quick I. The amino acid sequence obtained for samples from the peak labeled B in the thrombin Quick I chromatogram is given in Table I. The sequence X-Phe-Gly-Ser-Gly-Glu-Ala-Asp beginning at prothrombin residue 285 was identified in both peptides marked with an asterisk. The base line for the thrombin chromatogram dropped below zero absorbance at 35 min. Differences in elution position between the two chromatograms are attributed to slightly altered chromatographic conditions and/or residual trifluoroacetic acid in the sample.

Peptide Isolation. Peptides were isolated from regions A and B by preparative-scale chromatography and rechromatographed at pH 6.5. Chromatograms for the rechromatography of regions A and B are shown in Figures 2 and 3, respectively. Comparison of the two sets of chromatograms

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

cycle	prothrombin sequence position	thrombin		thrombin Quick I		
		amino acid identified	pmol	amino acid identified	pmol	pmol
1[b]	373					
2	374	Phe	120	Phe	99	>200
3	375	Thr	32	Thr	19	112
4	376	Glu	76	Glu	53	>180
5	377	Asn	64	Asn	52	141
6	378	Asp	52	Asp	31	144
7	379	Leu	47	Leu	45	146
8	380	Leu	46	Leu	38	131
9	381	Val	32	Val	23	84
10	382	Arg	17	X[c]		
11	383	Ile	22	Ile	19	45
12	384	Gly	18	Gly	14	54
13	385	Lys	13	Lys	11	23
14		none		none		

[a] Results are given for sequencing of peptide labeled A in Figure 2 and for duplicate runs of peptide labeled B in Figure 3. Sequence position numbers according to Degen et al. (1983). [b] Amino acid in cycle 1 is expected to be asparagine linked to carbohydrate. [c] PTH-amino acid was identified as Gln or (carboxymethyl)cysteine. See Figure 4 for identification of this residue.

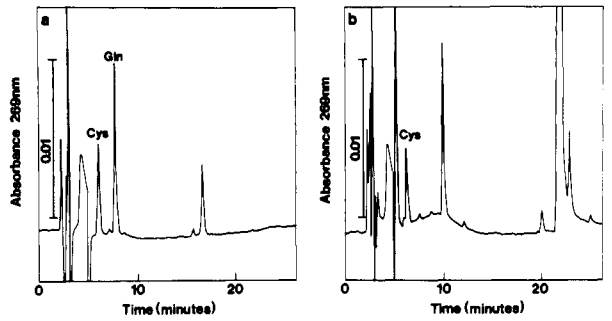

FIGURE 4: Identification of PTH-(carboxymethyl)cysteine. (a) Chromatographic separation of known samples of PTH-(carboxymethyl)cysteine, labeled Cys, and PTH-Gln, labeled Gln, by adjusting the pH of the initial chromatography buffer to 4.5 (see Experimental Procedures for further details). (b) Identification of PTH-(carboxymethyl)cysteine, labeled Cys, at position 382 from resequencing the peptide from thrombin Quick I labeled B in Figure 3.

shows a single peptide missing from region A in thrombin Quick I and the appearance of a new peptide in region B.

Identification of the Site of Amino Acid Substitution. The peptide from thrombin that was missing in thrombin Quick I was sequenced, as was the new peptide appearing in thrombin Quick I. These results with the associated recoveries of PTH-amino acids are shown in Table I and indicate that the altered residue in thrombin Quick I is Arg-382 of the human prothrombin sequence (Butkowski et al., 1977; Degen et al., 1983). No alternate sequences were detected in sequencing either the normal or the abnormal peptide.

Identification of Cys-382 in Thrombin Quick I. The elution position of the PTH-amino acid present at position 382 in thrombin Quick I corresponded to either glutamine or (carboxymethyl)cysteine. The altered chromatographic procedure that was used to distinguish between these two amino acids was verified by sequencing residues 342–350 of prothrombin Quick I with identification of Gln-344 and Cys-348. Chromatograms of the standards, PTH-(carboxymethyl)cysteine and PTH-glutamine, and for the sequencer cycle corresponding to residue 382 in thrombin Quick I are shown in Figure 4. Results from the preceding and following cycles confirmed that the peak corresponding to PTH-(carboxymethyl)cysteine was unique to the cycle containing residue 382. For this sequencer run, the recovery of the cysteine derivative was estimated at 60 pmol where the recovery of PTH-Phe in cycle 2 was 250 pmol (for comparison, see results for thrombin Quick I in Table I). The altered properties of thrombin Quick I may thus be explained by the replacement of Arg-382 by Cys.

Table II: Relative Activity of Thrombin Quick I[a]

substrate	act. of TQI/ act. of thrombin
benzoylarginine ethyl ester	0.74[b]
tos-Gly-Pro-Arg-*p*-nitroanilide	0.55[b]
fibrinogen Aα chain	0.012[b]
platelet aggregation	0.017[c]
prostacyclin release	0.024[c]

[a] The activity of thrombin Quick I (TQI) compared to that of thrombin in parallel experiments. [b] Results are for k_{cat}/K_m from Henriksen and Owen (1987). [c] Relative activity in platelet aggregation was determined by comparing the time from enzyme addition to initiation of aggregation. Prostacyclin release was from primary cultures of human umbilical vein endothelial cells. Results from Henriksen and Brotherton (1983).

Discussion

The results presented above have identified Arg-382 in the human prothrombin sequence as the site of substitution in thrombin Quick I. The substitution by Cys is consistent with a point mutation in a prothrombin gene of the affected individual. The decreased positive charge on thrombin Quick I that is predicted from its elution position from sulfopropyl-Sephadex is explained by this substitution (Henriksen & Owen, 1987). The amino acid composition of thrombin Quick I (Henriksen, 1985) and the identical chromatographic behavior of the other peptides obtained by lysyl endopeptidase hydrolysis of thrombin and thrombin Quick I are consistent with the absence of other changes in the primary structure of thrombin Quick I.

Arg-382 follows the carboxyl terminus of the B insertion loop when the amino acid sequence of thrombin is aligned with the bovine α-chymotrypsin sequence (Elion et al., 1977). This residue corresponds to an Arg in trypsin, but is substituted by Val in chymotrypsin. The primary structure for this region of thrombin is shown in Figure 5. In the three-dimensional structure of bovine trypsinogen, this Arg is located distal to the catalytic site, but on the same aspect of the molecule. If it is assumed that the tertiary structure of trypsinogen is conserved in thrombin, the location of this mutant residue is consistent with the observation that the activity of thrombin Quick I is minimally altered with respect to low molecular weight substrates, but is markedly decreased in the release of fibrinopeptide A, aggregation of platelets, and stimulation of

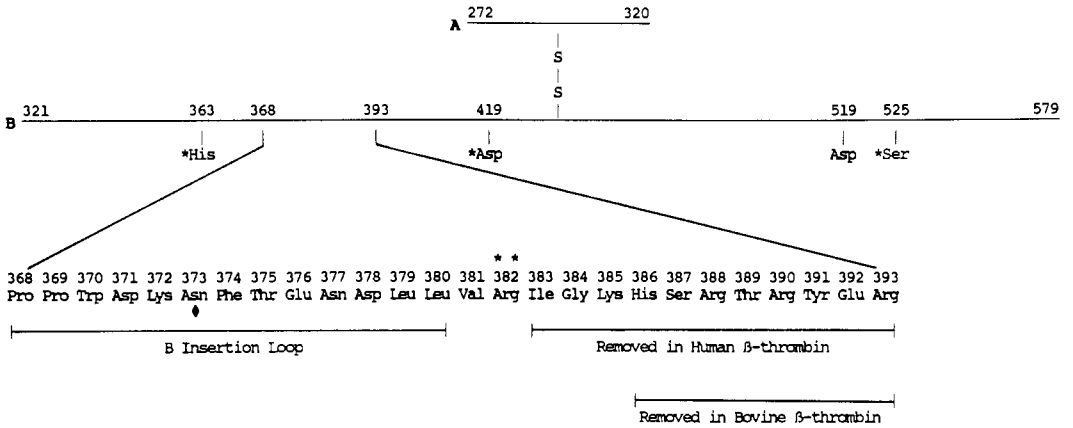

FIGURE 5: Diagram of human thrombin indicating the primary structure in the region of substitution in thrombin Quick I. The catalytic triad residues are indicated by one asterisk. Arg-382 which is replaced by Cys in thrombin Quick I is marked by two asterisks. Asp-519 is located at the base of the substrate binding pocket. The site of carbohydrate attachment is indicated by (♦). See text for references and discussion of structural features.

prostacyclin release from human umbilical vein endothelium (Table II). These latter three physiological reactions which involve interaction with a large protein substrate or a cellular surface might be expected to involve an extended region of interaction on the thrombin surface.

Evidence for the existence of a fibrinogen specificity site, extrinsic to the catalytic site, in thrombin has been summarized (Fenton, 1981). Further evidence for this specificity site comes from studies with thrombomodulin as a competitive inhibitor of the thrombin-catalyzed release of fibrinopeptides from fibrinogen and fibrinogen fragments (Jakubowski et al., 1986; Hofsteenge et al., 1986; Hofsteenge & Stone, 1987). More recently, studies of direct binding between thrombin and fragments of fibrinogen have identified fibrinogen α-chain residues 17–78 as containing a strong binding site for thrombin (Vali & Scheraga, 1988). The results of structural studies on degraded forms of thrombin which retain activity toward low molecular weight substrates but show a marked decrease in fibrinogen clotting activity indicate that prothrombin residues 386–393[2] are deleted in bovine β-thrombin (Lundblad et al., 1979) and that residues 383–393 are deleted in human β-thrombin with the additional loss of residues 444–474 in human γ-thrombin (Boissel et al., 1984). Defects of approximately 50-fold in both K_M and k_{cat} for release of fibrinopeptide A from fibrinogen by human γ-thrombin (Lewis et al., 1987) suggest the involvement of one or both of the deleted peptide regions in determining the specificity toward fibrinogen. For a form of thrombin, β_T-thrombin, in which a single hydrolysis occurs following residue 393, but where no peptide is lost, the K_M for release of fibrinopeptide A from fibrinogen is increased about 2-fold with an approximately 40-fold decrease in k_{cat}. Toward low molecular weight substrates of thrombin, β_T-thrombin had a 3-fold increase in K_M without a change in k_{cat}. For another derivative of thrombin, hydrolyzed specifically following residue 470, the hydrolysis of low molecular weight substrates was essentially unaffected, and there was less than a 2-fold decrease in k_{cat} for release of fibrinopeptide A (Hofsteenge et al., 1988). Although this latter study does not identify a region of thrombin critical for fibrinogen binding, it does indicate that the structure around residue 393 is critical for normal catalytic efficiency with respect to fibrinogen. When the human and bovine thrombin sequences are compared, the peptides comprising residues 378–393 and 455–463 are identical and also contain several residues that are conserved among serine proteases in general (Degen et al., 1983; MacGillivray & Davie, 1984). The previous functional characterization of thrombin Quick I suggested that the alteration in this molecule involves a specific fibrinogen binding site, extrinsic to the catalytic site, and that this defect is communicated to the catalytic site as evidenced by defects in both K_M and k_{cat} for the release of fibrinopeptide A (Henriksen & Owen, 1987). The results of the present study suggest that the fibrinogen specificity site includes Arg-382, a residue that is retained in both β- and γ-thrombins (see Figure 5).

Two chemically modified derivatives of thrombin with decreased fibrinogen clotting activity have been characterized with respect to the site of modification. Human thrombin, in the presence of heparin, is modified by pyridoxal phosphate at two sites in the B chain, Lys-341 and Lys-385 (Meade et al., 1987). Steady-state kinetic parameters for fibrinopeptide A release have not be determined for this derivative. However, the activity of phosphopyridoxylthrombin toward low molecular weight substrates was nearly identical with that of unmodified thrombin (Griffith, 1979). The modification by tetranitromethane of Tyr-391 and/or Tyr-405 in bovine thrombin results in a thrombin preparation in which the K_M for release of fibrinopeptide A from fibrinogen is unaltered, but k_{cat} is reduced to about one-third of that for unmodified bovine thrombin (Lundblad et al., 1988).

Thrombin Tokushima is the only other dysthrombin for which a primary structural defect has been identified. In this case, Arg-418 is replaced by Trp. Although Miyata et al. (1987a) concluded that this amino acid substitution was at a substrate binding site, they also reported that all catalytic activity of this dysthrombin was reduced including an increased K_M and a decreased k_{cat} for the substrate Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide. Arg-418 is adjacent to the conserved Asp residue of the catalytic triad. The primary structure defect in two other dysprothrombins has been determined. In both prothrombin Barcelona and prothrombin Madrid, the defect is at the factor Xa cleavage site between fragment 2 and prethrombin 2 where Arg-271 is replaced by Cys (Rabiet et al., 1986; Rabiet et al., 1987).

The substitution of Cys for Arg in thrombin Quick I is expected to result from a point mutation in which the codon, CGC, for Arg-382 (Degen et al., 1983) is converted to TGC. It is reported that cytosine in the sequence CpG is highly methylated and that this may result in making this sequence

[2] All sequence numbers refer to the human prothrombin sequence. The bovine prothrombin sequence has three additional residues in the amino-terminal activation fragments so that the actual position of bovine thrombin residues is the number indicated +3.

a "hotspot" for mutations, with C being replaced by T (Barker et al., 1984). Arg to Cys mutations have been reported in several other proteins of the coagulation pathway. These include the antithrombin III mutants Toyama (Koide et al., 1984), Tours (Duchange et al., 1987), Alger (Brunel et al., 1987), and Northwick Park (Erdjument et al., 1988) as well as fibrinogens Metz (Southan et al., 1982), Zurich I (Southan et al., 1982), Bergamo I (Reber et al., 1985), Osaka (Miyata et al., 1987b), Kawaguchi (Miyata et al., 1987b), Schwarzach (Henschen et al., 1983), Tochigi (Yoshida et al., 1988), and Stony Brook (Galanakis & Henschen, 1985). The same mechanism could also explain the Arg to Trp mutation in prothrombin Tokushima (Miyata et al., 1987a) and the reported mutation of the Arg codon to a stop codon in multiple cases of hemophilia A (Youssoufian et al., 1986).

The results presented here identify residue 382 in human prothrombin as essential for determining the specificity of thrombin toward fibrinogen and also in the cellular responses of platelet aggregation and prostacyclin release. As in other studies of this type, it is not possible, from the data available, to distinguish whether this residue is directly involved in specific binding interactions or is critical only for maintaining the structure of the binding site at another location in the enzyme. Resolution of this question will require additional investigation and would benefit from knowledge of the tertiary structure of this enzyme.

ACKNOWLEDGMENTS

We thank V.A. for donation of plasma and the staff of the Elmer L. DeGowin Memorial Blood Center at The University of Iowa Hospitals and Clinics, Iowa City, IA, for assistance in collection of plasma. Xaviar Villarreal operated the peptide sequencer, and Richard Jenny provided helpful advice in the operation of instrumentation. Figures were prepared by Trish M. Warshaw.

Registry No. Arg, 74-79-3; Cys, 52-90-4; prothrombin Quick, 117183-17-2; thrombin Quick I, 117183-22-9; thrombin Quick II, 117183-23-0; thrombin, 9002-04-4.

REFERENCES

Barker, D., Schafer, M., & White, R. (1984) *Cell (Cambridge, Mass.) 36*, 131–138.

Benson, J. R., & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 619–622.

Boissel, J. P., Le Bonniec, B., Rabiet, M. J., Labie, D., & Elion, J. (1984) *J. Biol. Chem. 259*, 5691–5697.

Brunel, F., Duchange, N., Fischer, A. M., Cohen, G. N., & Zakin, M. M. (1987) *Am. J. Hematol. 25*, 223–224.

Butkowski, R. J., Elion, J., Downing, M. R., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 4942–4957.

Degen, S. J. F., MacGillivray, R. T. A., & Davie, E. W. (1983) *Biochemistry 22*, 2087–2097.

Duchange, N., Chais, J.-F., Cohen, G. N., & Zakin, M. M. (1987) *Thromb. Res. 45*, 115–121.

Elion, J., Downing, M. R., Butkowski, R. J., & Mann, K. G. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 97–111, Ann Arbor Science Publishers, Ann Arbor, MI.

Erdjument, H., Lane, D. A., Panico, M., Di Marzo, V., & Morris, H. R. (1988) *J. Biol. Chem. 263*, 5589–5593.

Fenton, J. W., II (1981) *Ann. N.Y. Acad. Sci. 370*, 468–495.

Galanakis, D. K., & Henschen, A. (1985) in *Fibrinogen, Structural Variants and Interactions* (Henschen, A., Hessel, B., McDonagh, J., & Saldeen, T., Eds.) Vol. 3, pp 207–212, de Gruyter, Berlin.

Griffith, M. J. (1979) *J. Biol. Chem. 254*, 3401–3406.

Henriksen, R. A. (1985) Ph.D. Thesis, University of Iowa, Iowa City, IA.

Henriksen, R. A., & Brotherton, A. F. A. (1983) *J. Biol. Chem. 258*, 13717–13721.

Henriksen, R. A., & Owen, W. G. (1987) *J. Biol. Chem. 262*, 4664–4669.

Henriksen, R. A., Owen, W. G., Nesheim, M. E., & Mann, K. G. (1980) *J. Clin. Invest. 66*, 934–940.

Henschen, A., Kehl, M., & Deutsch, E. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1747–1751.

Hofsteenge, J., & Stone, S. R. (1987) *Eur. J. Biochem. 168*, 49–56.

Hofsteenge, J., Tagucki, H., & Stone, S. R. (1986) *Biochem. J. 237*, 243–251.

Hofsteenge, J., Braun, P. J., & Stone, S. R. (1988) *Biochemistry 27*, 2144–2151.

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399–442.

Jakubowski, H. V., Kline, M. D., & Owen, W. G. (1986) *J. Biol. Chem. 261*, 3876–3882.

Koide, T., Odani, S., Takahashi, K., Ono, T., & Sakuragawa, N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 289–293.

Lewis, S. D., Lorand, L., Fenton, J. W., II, & Shafer, J. A. (1987) *Biochemistry 26*, 7597–7603.

Lundblad, R. L., Kingdon, H. S., & Mann, K. G. (1976) *Methods Enzymol. 45*, 156–176.

Lundblad, R. L., Noyes, C. M., Mann, K. G., & Kingdon, H. S. (1979) *J. Biol. Chem. 254*, 8524–8528.

Lundblad, R. L., Noyes, C. M., Featherstone, G. L., Harrison, J. H., & Jenzano, J. W. (1988) *J. Biol. Chem. 263*, 3729–3734.

MacGillivray, R. T. A., & Davie, E. W. (1984) *Biochemistry 23*, 1626–1634.

Mann, K. G., & Lundblad, R. L. (1987) in *Hemostasis and Thrombosis, Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., & Salzman, E. W., Eds.) pp 148–161, Lippincott, Philadelphia, PA.

Meade, J. B., Noyes, C. M., & Church, F. C. (1987) *Thromb. Haemostasis 58*, 505.

Miyata, T., Morita, T., Inomoto, T., Kawauchi, S., Shirakami, A., & Iwanaga, S. (1987a) *Biochemistry 26*, 1117–1122.

Miyata, T., Terukina, S., Matsuda, M., Kasamatsu, A., Takeda, Y., Murakami, T., & Iwanaga, S. (1987b) *J. Biochem. (Tokyo) 102*, 93–101.

Quick, A. J. (1974) *The Hemorrhagic Diseases and the Pathology of Hemostasis*, Charles C. Thomas, Springfield, IL.

Quick, A. J., Pisciotta, A. V., & Hussey, C. V. (1955) *Arch. Intern. Med. 95*, 2–14.

Rabiet, M. J., Furie, B. C., & Furie, B. (1986) *J. Biol. Chem. 261*, 15045–15048.

Rabiet, M. J., Furie, B. C., & Furie, B. (1987) *Thromb. Haemostasis 58*, 313.

Reber, P., Furlan, M., Beck, E. A., Finazzi, G., Buelli, M., & Barbui, T. (1985) *Thromb. Haemostasis 54*, 390–393.

Southan, C., Henschen, A., & Lottspeich, F. (1982) in *Fibrinogen, Recent Biochemical and Medical Aspects* (Henschen, A., Graeff, H., & Lottspeich, F., Eds.) pp 153–166, de Gruyter, New York.

Vali, Z., & Scheraga, H. A. (1988) *Biochemistry 27*, 1956–1963.

Yoshida, N., Ota, K., Moroi, M., & Matsuda, M. (1988) *Blood 71*, 480.

Youssoufian, H., Kazazian, H. H., Jr., Phillips, D. G., Arnois, S., Tsiftis, G., Brown, V. A., & Antonarakis, S. E. (1986) *Nature (London) 324*, 380–382.