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HIGH AFFINITY BINDING OF HUMAN AND BOVINE THROMBINS TO *p*-CHLOROBENZYLAMIDO-ϵ-AMINOCAPROYL AGAROSE

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Summary

Human thrombin (EC 3.4.21.5) binds tightly to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose, and is not eluted by 2 M NaCl at pH 8. Its zymogen, human prothrombin, does not bind to the same adsorbent. 2 M NaCl partially elutes DFP-treated thrombin. For native human and bovine thrombins, protein and activity are quantitatively eluted by 25% dioxane, and upon rechromatography the active human enzyme exhibits the same binding properties. Equally tight binding of human thrombin occurs with derivatives of the *m*- and *p*-chlorobenzylamines. With the *o*-chloro derivative or benzylamine itself insolubilized to ϵ-aminocaproyl agarose, thrombin is eluted by high ionic strength.

Bovine trypsin and bovine factor Xa bind less tightly than thrombin to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose, being eluted by high ionic strength. It is proposed that the specific thrombin adsorption is related to a secondary binding site of high affinity and with hydrophobic properties. This site is not available in the zymogen. Furthermore, the less specific protease, trypsin, and the more specific protease, factor Xa, lack this binding site.

Introduction

Bovine thrombin (EC 3.4.21.5) is adsorbed to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose which enabled further purification from inactive contaminant protein [1]. The same adsorption has been used in the purification of human thrombin [2]. The amino group of this ligand is acylated, however, such that its interaction with thrombin cannot be explained by active site binding as with the inhibition of trypsin by benzamidine where the charged amidino group binds to Asp_{189} [3]. The *p*-chlorobenzyl moiety is strongly

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hydrophobic, suggesting a nonpolar interaction with the thrombin molecule. Hydrophobic interactions appear to be important to the inhibition of thrombin by low molecular weight compounds [4]. In comparative studies, however, no inhibitor was found with a clear preference for thrombin over trypsin [5].

Since the binding of thrombin to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose was remarkably stable at high ionic strength, the present study was undertaken to examine the hydrophobic nature of this interaction. The presence and position of the chloro group have been investigated as were properties of native and DFP-treated thrombin. A qualitative specificity for thrombin was demonstrated by comparing binding of prothrombin, trypsin and factor Xa.

Materials and Methods

Proteins. Human thrombin was generously provided by Dr. John Fenton, Research Laboratories, State Department of Health, Albany, New York, 12201; as an extrinsically activated human Cohn fraction III paste which had been chromatographed over a CG-50 resin as previously described [6,7]. This material was 85—95% active by *p*-nitrophenyl-*p'*-guanidobenzoate active site titration [8]. Samples were shipped on dry ice and were stored at −70° C in 0.75 M NaCl until use. Resin purified bovine thrombin was prepared as previously described [9]. Bovine trypsin was purchased from Sigma Chemical Company (grade 1) St. Louis, Mo., and bovine factor X_1a was a gift of Dr. Kazuo Fujikawa as Russell's viper venom activated product [10,11].

Human prothrombin was prepared from human cryosupernate plasma using barium citrate absorption-elution [12], DEAE-Sephadex chromatography and chromatography over heparin agarose. The latter two steps were as previously described for bovine factor IX [13], using the void volume fraction from the last step. It contained no detectable factor IX, X or VII and was homogeneous by immunoelectrophoresis and on 7.5% polyacrylamide-sodium dodecyl sulfate gel electrophoresis (apparent molecular weight of 71 000). Samples were 0.4—0.8 mg/ml dialyzed against 0.75 M NaCl prior to use. An extinction coefficient of Kisiel and Hanahan, 13.8 [14], was used to determine prothrombin concentration.

Other materials. Benzylamine and 2,3- and 4-chlorobenzylamines were purchased from the Aldrich Chem. Co. (Milwaukee, Wi.), ϵ-aminocaproic acid, dithiothreitol, sodium dodecyl sulfate and DFP came from Sigma Chem. Co. (St. Louis, Mo.). For DFP inhibition, 0.2 ml of 1 M stock solution (in redistilled isopropanol) was added to 15 mg human thrombin in 10 ml 0.38 M NaCl/60 mM NaH_2PO_4 (pH 8) and incubated for 40 min at 37° C at which point less than 1% clotting activity remained*. Sepharose 4B and G-25 Sephadex were purchased from Pharmacia (Piscataway, N.J.), and 1-cyclohexyl-3/2-morpholino/4/ethyl/carbodiimide came from Kand K. Laboratories (Plainview, N.Y.). Cyanogen bromide was purchased from Matheson, Coleman and Bell (Norwood, Ohio), and 1,4 dioxane was a product of J.T. Baker (Phillipsburg, N.J.). Other chemicals and solvents used were of the highest grade available.

* In 0.75 M NaCl, inactivation was much slower.

Clotting assays. Thrombin clotting activities were determined as previously described [9] and factor Xa was assayed by a modification of the method of Bachman [15,16,10] using factor X-deficient bovine plasma (Seitz filtered), Centrolex-P as the phospholipid source and Veronal buffer for sample dilution. Preincubation at 37°C for 30 s was followed by the addition of 35 mM $CaCl_2$.

Preparation of adsorbent. *p*-Chlorobenzylamido-ϵ-aminocaproyl agarose was prepared by attaching ϵ-aminocaproic acid to CNBr-treated agarose and then coupling the inhibitor-amine by a carbodiimide reaction. Agarose was activated by CNBr according to Cuatrecasas [17], then ϵ-aminocaproic acid, in a ratio of 50 μmol ligand per ml packed agarose, was added. In a typical experiment, 40 ml Sepharose 4B was reacted with 5—10 g CNBr (dissolved in 5 ml 5,10-*N*,*N*-dimethylformamide) and, following a 0.1 M $NaHCO_3$ (pH 9) wash, 2 mmol ligand in 40 ml bicarbonate buffer was rapidly added to the activated agarose and reacted overnight at 4°C with gentle stirring. After thorough washing with 1 M NaCl, the product gave a light orange color reaction to picrylsulfonic acid [16,17]. Agarose was then washed with 50% dioxane and taken up in an equal volume (40 ml) 50% dioxane containing 1 g (8 mmol) *p*-chlorobenzylamine. The pH was adjusted to 4.7 with 6 N HCL and 1 g (2.3 mmol) 1-cyclohexyl-3/2-morpholino/4/ethyl/carbodiimide in 4 ml 50% dioxane was slowly added over 5 min at 25°C. The reaction was stirred 12 h and the gel was then washed on a column with 50% dioxane until the absorbancy (280 nm) was zero (about 100 ml). The substituted gel was then washed with 2 M NaCl/0.1 M NaH_2PO_4 (pH 8). It was stored at 4°C in 30 mM sodium azide. The carbodiimide conditions were adapted from the coupling of estradiol to amino-ethyl agarose, according to Cuatrecasas and Anfinsen [18].

Chromatography of human thrombin. Human thrombin in 0.75 M NaCl was first diluted with one-tenth volume to a final concentration of 30 mM NaH_2PO_4 (pH 8). The adsorbent gel with a column height of 3—4 cm, was equilibrated with the same phosphate/NaCl buffer. Flow rates were 1 ml per min and 1—3 ml fractions were collected at 23°C. All chromatography experiments were run at least three times with essentially identical results. Simultaneous controls were run with native human thrombin over *p*-chlorobenzylamido-ϵ-aminocaproyl agarose.

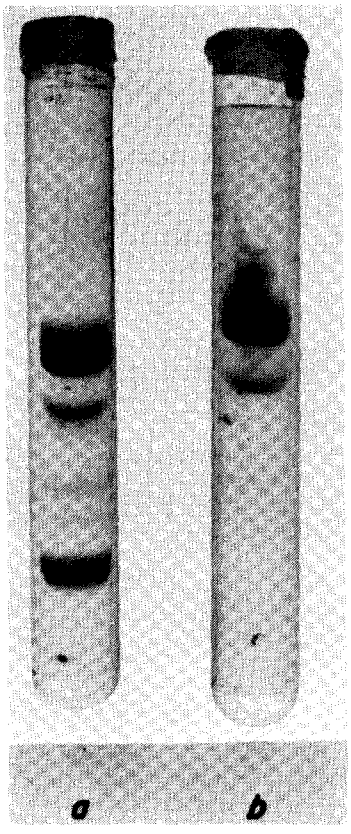
Gel electrophoresis. Polyacryamide gel electrophoresis in 0.5% sodium dodecyl sulfate was performed by the technique of Weber and Osborn [19]. Gels were 70—100 × 3 mm and 20—50 μg protein were applied and run for approximately 4—5 h at 8 mA per gel. They were stained with Coomassie brilliant blue, R-250, destained in 7% acetic acid/20% methanol and then stored in 7% acetic acid. For molecular weight estimates, a mixture of the following standards was used: phosphorylase (100 000), bovine serum albumin (69 000), ovalbumin (43 000) and carbonic anhydrase (27 000).

Extinction coefficient. A sample containing 1 mg per ml human thrombin following dioxane elution was dialyzed extensively against 0.75 M NaCl/10 mM NaH_2PO_4, pH 6.4. Protein concentration was determined by fringe pattern in the analytical ultracentrifuge, and absorbtivity in a Cary model 15 recording spectrophometer according to the method of Babul and Stellwagen [20]. The extinction coefficient of thrombin was $E^{1\%}_{280nm} = 19.17$ and this value was used to determine protein concentrations for human thrombin. The correction

for light scattering by absorbancy at 320 nm was less than 5%. This value is comparable to that of bovine thrombin [21], but is higher than that found for a less pure human preparation [22].

Results

Examination of human thrombin on 8.5% polyacrylamide gels revealed a heavy band and variable amounts of faster, low molecular weight peptide material (Fig. 1a). After adsorption to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose, only the heavy band with smaller amounts of one additional faster component (Fig. 1b) were observed. For non-reduced samples, the major band corresponded to a molecular weight of 37 000 when run with the standard proteins. After reduction with dithiothreitol, a single heavy band corresponded to a molecular weight of 33 000.



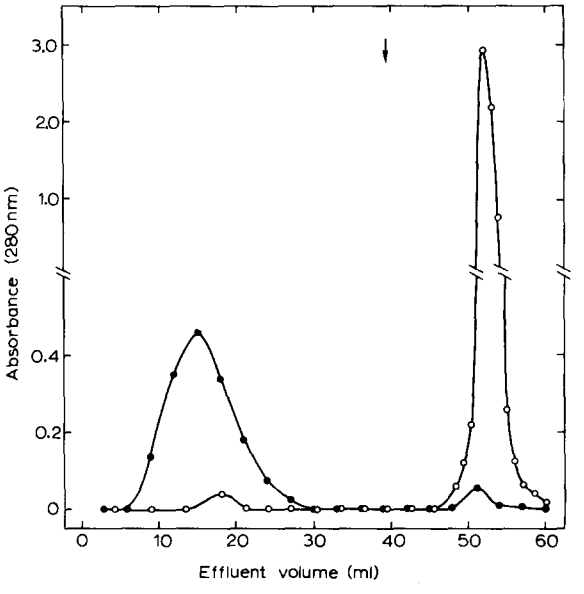


Fig. 1. Purity of human thrombin. Sodium dodecyl sulfate/8.5% polyacrylamide gel electrophoresis of starting material (85% pure by titration) and material chromatographed over the specific adsorbent, 50 μg protein on each gel.

Fig. 2. Comparison of trypsin and thrombin. Chromatography on a 4 cm × 1 cm *p*-chlorobenzylamido-ϵ-aminocaproyl agarose column. Human thrombin (open circles) or bovine trypsin (closed circles) 5 mg in 2 ml 0.75 M NaCl/30 mM NaH PO_4, were applied separately and washed with 15 ml 0.3 NaH_2PO_4 (pH 8), 20 ml 2 M NaCl/0.1 M NaH_2PO_4 (pH 8) and 20 ml 25% dioxane in 0.75 M NaCl/75 mM NaH_2PO_4 (pH 8). Flow rate was 1 ml/min. Arrow indicates point of application of the dioxane.

As shown in Figs. 2 and 3, as much as 5 mg human thrombin in 0.75 M NaCl (pH 8) bound completely to as little as 4 ml substituted agarose and was not eluted when the salt concentration was increased to 2 M. The 1—2% of protein which eluted in the thrombin salt wash contained no fibrinogen clotting activity*. In preliminary experiments, 1 M benzamidine only eluted about 10% human thrombin and no protein was removed by 10 mM *p*-toluenesulfonic acid. Slow desorption occurred at pH 6, with either low or high ionic strength. This presumably reflects the lower limit of pH stability of thrombin. Recovery of protein and activity was quantitative with 25% dioxane. The dioxane was removed by gel filtration through G-25 Sephadex in 0.75 M NaCl**. Following gel filtration, human thrombin exhibited the same binding properties on rechromatography and was again quantitatively removed by dioxane. Bovine thrombin was also quantitatively recovered by the dioxane elution; the 44% inactive, contaminant protein recovered in the salt wash before dioxane elution agrees well with the degree of purification previously described [1]. Either thrombin protein could also be quantitatively recovered in a denatured form by 1 M acetic acid elution (not shown).

The role of the *p*-chloro group on the ligand was then investigated. 2 mg of human thrombin were applied to a 4.5 cm × 1 cm column of benzylamido-ε-aminocaproyl agarose. A trace of protein eluted with a 0.3 M sodium phos-

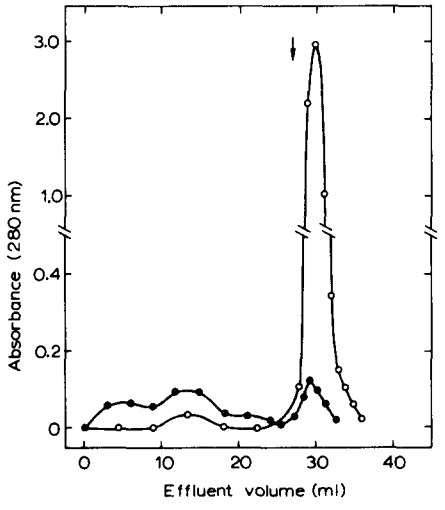


Fig. 3. Comparison of factor Xa and thrombin. Chromatography on a 3 cm × 1 cm *p*-chlorobenzylamido-ε-aminocaproyl agarose column. Human thrombin (open circles, 5 mg) or bovine factor Xa (closed circles, 1.5 mg) in 3 ml 0.1 M Tris (pH 7.5) were applied separately and washed with 5 ml 0.3 M NaH_2PO_4 (pH 7.8), then 10 ml each of 0.75 M NaCl/75 mM NaH_2PO_4 (pH 7.8), 2 M NaCl/0.1 M NaH_2PO_4 (pH 7.8) and (at arrow) 25% dioxane in NaCl and phosphate buffer as in figure 2.

* Presence of clotting activity was only seen when additional protein or less gel were used or if the gel had been used several times. In the latter case, up to 10% of a 5 mg load gradually eluted during extensive 2 M NaCl washes, prior to dioxane elution.

** Protein precipitated upon thawing if frozen in the presence of dioxane. Thrombin was readily eluted by dioxane alone but 0.75 M NaCl was routinely included in the dioxane elution buffer to insure stability upon gel filtration.

phate (pH 8) wash but contained no clotting activity. With 20 ml 1.5 M NaCl/0.15 M sodium phosphate (pH 8) wash ($I \simeq 2$), 70% of the protein and clotting activity were eluted in a broad, continuous peak. Acetic acid (1 M) was added at that point and the remaining 30% of protein eluted in a sharp peak. Ortho and *m*-chloro derivatives were then studied. With the former, human thrombin was eluted by high ionic strength identically to the unsubstituted benzylamine derivative; whereas the *m*-chloro derivative bound thrombin as tightly as in the *p*-chloro experiments, requiring 25% dioxane.

To examine the properties of the enzyme necessary for binding, DFP-inhibited thrombin and then prothrombin were studied. Thrombin was treated with DFP until less than 1% activity remained (see methods), and applied to a 3.5 cm × 1 cm column of *p*-chlorobenzylamido-ε-aminocaproyl agarose. The column was washed with 10 ml 0.75 M NaCl in 75 mM sodium phosphate (pH 7.8), then 20 ml 2 M NaCl/0.15 M sodium phosphate (pH 7.8). For a 5 mg sample, 3 mg were slowly eluted beginning with the first wash and the remaining 2 mg were then recovered as a sharp protein peak with 25% dioxane elution. With one-half the load, 70% was eluted by dioxane as was 90% of a 1.3 mg sample. Human prothrombin was likewise applied to the specific adsorbent, but, as shown in Fig. 4, failed to bind when applied in 0.75 M NaCl at pH 8. Although no detectable fibrinogen clotting or thrombin protein was observed in the starting prothrombin preparation, it is of interest that trace clotting activity (about 0.03 NIH units/ml) was found in the dioxane eluate peak. Sodium dodecyl sulfate gels of this fraction revealed a single faint protein band with intermediate migration to that of prothrombin and thrombin. Mixtures of thrombin and prothrombin were then chromatographed and demonstrated separation of the enzyme from its zymogen due to the tight adsorption of the former (not shown).

Bovine trypsin and factor Xa were compared with thrombin as they represent serine proteases of differing specificities. The results with trypsin are

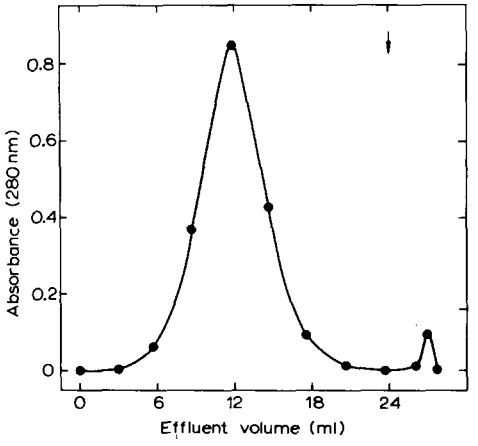


Fig. 4. Human prothrombin. Elution from a 4 cm × 1 cm column of *p*-chlorobenzylamido-ε-aminocaproyl agarose. 6 ml containing 5 mg prothrombin in 0.75 M NaCl/30 mM NaH_2PO_4 (pH 8), were applied. The column was then washed with 10 ml each of 0.75 M NaCl/75 mM NaH_2PO_4 (pH 8), 2 M NaCl/0.1 M NaH_2PO_4 (pH 8) and (at arrow) 25% dioxane as in Fig. 2.

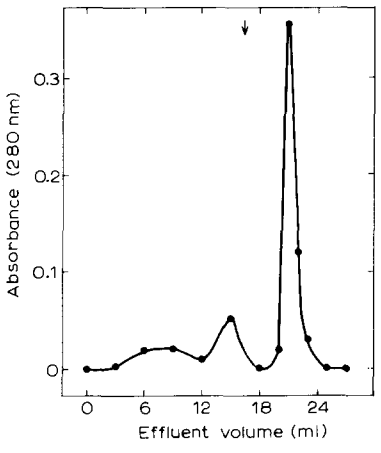


Fig. 5. Chromatography of a mixture of human thrombin and bovine factor Xa. Human thrombin (0.3 mg) and bovine factor Xa (0.1 mg) were mixed (2.1 ml), and added to a *p*-chlorobenzylamido-ε-aminocaproyl agarose column (4 cm × 1 cm). Washes were as in Fig. 4, including 5 ml each of 0.75 M NaCl, 2 M NaCl and (at arrow) dioxane/NaCl buffers.

shown in Fig. 2. The trypsin elution (solid circles) was similar to that of prothrombin, with essentially no binding when applied (ionic strength ≃1). In two other experiments about 20% of the trypsin protein was eluted in 0.75 M NaCl/75 mM NaH_2PO_4 (pH 8) and the balance with 20 ml 2 M NaCl/0.1 M NaH_2PO_4 (pH 8) (not shown).

The elution profile of bovine factor Xa was then compared with that of thrombin (Fig. 3). Again, the majority of the protein which contained potent factor Xa activity was eluted by the salt washes at pH 8 (ionic strength ≃1,

TABLE I

RELATIVE BINDING TO *p*-CHLOROBENZYLAMIDO-ε-AMINOCAPROYL AGAROSE

5 mg samples were used except for bovine thrombin (3 mg) and factor Xa (1.5 mg). Data was taken from experiments detailed in Figs. 1—4 and, for DIP-thrombin and bovine thrombin, from the text. Bovine thrombin (resin purified) contained 44% inactive contaminant in the salt wash. Values for human thrombin and prothrombin exclude the 1—2% protein in the sample void or dioxane elute, respectively. In the former, the thrombin void contained no clotting activity whereas the prothrombin dioxane eluate contained an intermediate protein species on gel electrophoresis and a trace of clotting activity. For DFP-treated thrombin, a relative increase in dioxane eluate protein was seen with decreased sample size (see text).

Protein	Percent recovery		
	$I \simeq 1$[a]	$I \simeq 2$[b]	Dioxane
Thrombin (bovine)	0	0	100
Thrombin (human)	0	0	100
DIP-Thrombin (human)	0	10—60	40—90
Prothrombin (human)	100	0	0
Trypsin (bovine)	98	0	2
Factor Xa (bovine)	15	70	15

(a) Ionic strength of 0.87: 0.3 M NaH_2PO_4 (pH 8), or 0.98: 0.75 M NaCl/75 mM NaH_2PO_4 (pH 8).
(b) Ionic strength of 2.29: 2 M NaCl/0.1 M NaH_2PO_4 (pH 8).

then 2). To examine the ability of the specific adsorbent to separate thrombin and factor Xa, a mixture of these clotting enzymes (0.3 mg thrombin, 0.1 mg factor Xa) was applied to another column (Fig. 5). The salt washes gave 2 peaks of factor Xa activity without fibrinogen clotting and could account for essentially all the factor Xa absorbancy units applied. The dioxane elution (3rd peak) contained potent fibrinogen clotting activity, the equivalent absorbancy units of the applied thrombin and no evidence of factor Xa protein on sodium dodecyl sulfate gel electrophoresis.

Table I summarizes the relative binding of the above proteins to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose. It should be noted that only native thrombin remained completely bound before dioxane elution.

Discussion

The adsorption of bovine thrombin to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose resulted in additional purification of the enzyme from inactive contaminants. The binding to the resin was quite strong, however, and 1 M benzamidine was required to elute the enzyme. In the interaction of bovine thrombin with an insolubilized derivative of *p*- or *m*-aminobenzamidine, Schmer eluted with 0.2 M benzamidine [23] and Hixon and Nishikawa were able to elute with 50 mM benzamidine [24]. These results occurred despite the fact that *p*-aminobenzamidine is by 4-fold a better esterase inhibitor of thrombin than *p*-chlorobenzylamine [5]. Therefore, the tight binding of thrombin to the insolubilized derivative of *p*-chlorobenzylamine involves an alternative binding site.

In the present study, human thrombin was bound even more tightly to *p*-chlorobenzylamido-ϵ-aminocaproyl agarose than bovine as high concentrations of either benzamidine or NaCl failed to elute it. Using an insolubilized benzylamine or *o*-chlorobenzylamine, high ionic strength eluted the thrombin. The presence of the chloro group in the meta or para position was associated with binding that could no longer be broken by high ionic strength alone. Quantitative recovery of active human and bovine enzymes was readily obtained with 25% dioxane, a non-polar solvent (Table I). Since thrombin retains full activity in this solvent, the most reasonable explanation is that the insolubilized ligand bound to a hydrophobic site or "pocket" in the thrombin molecule*.

As prothrombin failed to bind, this particular thrombin binding site must have become exposed upon activation. Thrombin alkylated by DFP bound tightly to the adsorbent, although the capacity for the inhibited enzyme was decreased. In addition, one lower molecular weight band was purified with thrombin (Fig. 1), and this probably represents "β-thrombin", a degradation product which retains esterase activity [26]. Thus an intact enzyme is required for binding, but it is not clear if this binding is specifically at the active site.

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* This is opposed to acetic acid elution in which irreversible denaturation elutes the thrombin. The presence of the ϵ-aminocaproyl "spacer arm" greatly facilitated thrombin binding [1] and may contribute to the non-polar nature of the interaction. The present binding, however, differs from "hydrophobic chromatography", in which enzyme interaction with the spacer arm is disrupted by high ionic strength [25].

The serine proteases, trypsin, thrombin and factor Xa, share identical active site sequences and exhibit considerable homology [27]. Quantitative differences in the specificities of these enzymes have been noted in comparisons of inhibitors, either irreversible or competitive, or by studying esterase, peptidase or proteolytic activities. Some degree of specificity for the inhibition of thrombin by *p*-chlorobenzylamine was suggested by Markwardt et al. [5]. They found this compound to be by 50-fold a better thrombin esterase inhibitor than benzylamine. The inhibiting trypsin, *p*-chlorobenzylamine was less active than benzylamine. The *p*-chloro derivative was the only compound in a series of substituted benzylamines and benzamidines with a lower K_i for thrombin than trypsin. The presently described interaction of thrombin with the insolubilized derivative of *p*-chlorobenzylamine is qualitatively distinct from that of either the less specific protease, trypsin, or a more specific enzyme, factor Xa. Thrombin alone required a non-polar solvent for elution.

A hydrophobic site unique to thrombin has not previously been demonstrated, although there is evidence for non-polar interactions. Thrombin [28,29,30], like trypsin [31], will interact with neutral substrates and both enzymes prefer a hydrophobic residue adjacent to the amino terminus of the Arg-X [32,33]. Only thrombin demonstrated specificity for the subsequent amino-terminal residue. In examining protein substances, trypsin is by far the least specific. For thrombin, a non-polar residue (Val or Ala) is found in either of the two adjacent amino-terminal residues [34,35]. Factor Xa substrates frequently contain an acidic but no hydrophobic residue at this site*. Another potential hydrophobic site is a Phe binding site as this residue is found 8 positions from the carboxy-terminal Arg in fibrinopeptides A from several species [34,39]. A binding of this residue with thrombin, is conceivably a component of the enzyme-substrate complex. Indeed, *p*-chlorobenzylamido-ϵ-amino caproic acid did inhibit fibrinogen clotting activity [1]. Thus, the hydrophobic interaction may interfere with the active site or other substrate binding sites of thrombin, but at this time a relationship remains conjectural.

Nevertheless, the adsorption of thrombin by *p*-chlorobenzylamido-ϵ-aminocaproyl agarose represents a high affinity binding which is highly specific, and qualitatively differs from the interaction with its zymogen, trypsin and factor Xa. This binding is useful in the purification of thrombin. Furthermore, this technique can successfully separate thrombin from its zymogen or from factor Xa.

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* Factor Xa hydrolysis of the bond between the A and B chains of thrombin [36] and of oxidized trypsinogen [37] follows this pattern. Of interest, the shorter A chain of human thrombin [2] is best explained by a Lys_{13} to Arg replacement and an additional cleavage at Asn-Glu-Arg-Thr_{14} which is more consistent with a factor Xa cleavage than autolysis. Bovine factor Xa (without factor V), however, apparently failed to cleave this bond [38].

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