# Preparation of anhydrothrombin and characterization of its interaction with natural thrombin substrates

Kazuya HOSOKAWA*†, Tomoko OHNISHI*, Midori SHIMA‡, Masanori NAGATA† and Takehiko KOIDE*[1]

*Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Garden City, Hyogo 678-1297, Japan, †Fujimori Kogyo Co., 56 Imaikami-cho, Nakahara-ku, Kawasaki, Kanagawa 211-0067, Japan, and ‡Department of Pediatrics, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-0813, Japan

Thrombin is a serine proteinase that plays a key role in thrombosis and haemostasis through its interaction with several coagulation factors. Anhydrothrombin was prepared from PMSF-inactivated thrombin under alkaline conditions, and the folded anhydrothrombin was successfully recovered after dialysis in the presence of glycerol. Anhydro-derivatives of factor Xa, factor VIIa and activated protein C could also be prepared essentially by the same procedure. Anhydrothrombin retained affinity for various natural substrates of thrombin, including fibrinogen, factor VIII, factor XIII and protein C. In addition, these proteins were bound to anhydrothrombin–agarose in a reversible manner. The $K_d$ values for factor VIII, fibrinogen, factor XIII and protein C were $1.2 \times 10^{-8}$, $4.4 \times 10^{-8}$, $2.8 \times 10^{-7}$ and $8.1 \times 10^{-5}$ M, respectively. Thus thrombin substrates known to interact with the exosite I of thrombin demonstrated high affinity for anhydrothrombin. Furthermore, in the presence of $Na^+$, substantial enhancement of the association rate constant ($k_{ass}$) was observed for interactions of fibrinogen and factor VIII with anhydrothrombin. These results suggest that anhydrothrombin is useful in the purification of thrombin substrate proteins as well as in the investigation of detailed interactions between thrombin and these substrates in their activation or degradation processes.

Key words: factor VIII, factor XIII, fibrinogen.

## INTRODUCTION

Thrombin is a trypsin-like serine proteinase involved in the coagulation cascade. The primary role of thrombin is to proteolytically convert fibrinogen into fibrin and activate factor XIII to XIIIa. Factor XIIIa then crosslinks fibrin to produce a stabilized fibrin network. Furthermore, thrombin is an important catalyst of the coagulation cascade by converting factors V and VIII into factors Va and VIIIa, respectively [1–3]. Thrombin-mediated activation of the thrombin receptor 1 (PAR1) leads to a variety of cellular effects, including platelet aggregation, generation of phosphatidylserine for blood clotting [4] and tissue remodelling [5,6]. In the presence of thrombomodulin, thrombin functions as an anticoagulant by converting protein C into activated protein C. The latter then inactivates factors Va and VIIIa, by minor proteolysis, to factors Vi and VIIIi, respectively [7]. Thus thrombin interacts with a variety of coagulation proteins with a high degree of specificity and plays a key role in thrombosis and haemostasis.

Recently, procoagulant and anticoagulant activities of thrombin have been shown to be controlled by an allosteric switch through $Na^+$ binding to thrombin [8–11]. Thrombin is an allosteric enzyme existing in two forms, fast and slow, that differ widely in their specificities. The two forms are significantly populated *in vivo*, and an allosteric equilibrium can be affected by the binding of effectors and natural substrates. The fast form with $Na^+$ binding is a procoagulant, because it cleaves fibrinogen with higher specificity than the slow form, whereas the latter is an anticoagulant, because it preferentially activates protein C [8–11].

Thrombin consists of two polypeptides (A and B chains) that are linked by a single disulphide bond. The A chain consists of 49 residues, but there is no evidence thus far that the A chain is directly involved in the interactions of thrombin with other proteins. The 259-residue B chain that contains the catalytic domain is largely homologous with chymotrypsin and trypsin [12]. X-ray crystal structures of human thrombin and its complex with hirudin show that the thrombin molecule can be divided into several functional regions [13,14], among which active-site residues (Ser-195, His-57 and Asp-102), two anion-binding sites (exosites I and II) and two loops on the surface of the B chain are known to play an important role in its interaction with other proteins. Noteworthy in this regard is that exosite I has been reported to interact with a variety of substrates and inhibitors [15–17].

Anhydro-derivatives of chymotrypsin and trypsin have been prepared by eliminating a phenylmethylsulphonyl (PMS) residue from the PMS derivative under alkaline conditions. This converted the active-site serine into dehydroalanine [18,19]. The anhydrochymotrypsin and anhydrotrypsin, without catalytic activities, were reported to retain their binding properties similar to those of the native enzyme for both protein inhibitors and small substrate-like ligands [20–22].

In this study, we prepared completely refolded anhydrothrombin by using glycerol after the anhydro reaction. We then examined its interactions with thrombin substrates by affinity chromatography and by kinetic measurements by the surface plasmon resonance method, and showed that anhydrothrombin retained high affinity for several natural thrombin substrates, including fibrinogen, factor VIII and factor XIII.

## EXPERIMENTAL

### Materials

Human thrombin was prepared according to the method of Fenton [23]. PMSF, benzamidine and *p*-nitrophenyl-*p*′-amidinophenyl methanesulphonate were purchased from Wako Pure Chemical Co. (Osaka, Japan). Human factor XIII was purchased from Cosmo Bio Co. (Tokyo, Japan). Human factor VIII [24], fibrinogen [25] and antithrombin [26] were purified as

Abbreviations used: ChCl, choline chloride; PMS, phenylmethylsulphonyl; GdnHCl, guanidium hydrochloride; NHS, *N*-hydroxysuccinimide.

[1] To whom correspondence should be addressed (e-mail koide@sci.himeji-tech.ac.jp).

described previously. Human protein C was kindly provided by Dr W. Kisiel (University of New Mexico, Albuquerque, NM, U.S.A.). Benzamidine–agarose and Hi-trap NHS (*N*-hydroxysuccinimide)-activated columns were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Co. Thrombin activity was determined by using S-2238 as substrate. Protein concentrations were determined by absorbance measurements at 280 nm. Dehydroalanine content of anhydrothrombin was measured as described by Weiner et al. [27].

### Preparation of anhydrothrombin

A 7% methanolic solution of PMSF (30 $\mu$l) was added at intervals of 30 min to thrombin (60 mg in 100 ml of 5 mM sodium phosphate/0.1 M NaCl, pH 6.5) at 20 °C, until the thrombin activity decreased to $< 1\%$. The solution was then dialysed against the same buffer for 2 h at 4 °C. The resulting PMS-thrombin solution was diluted to 200 ml at 0 °C, followed by the addition of 10.5 ml of 1 M NaOH. The solution was then incubated for 12 min to complete the elimination reaction. To the resultant reaction mixture, 3 M NaCl and glycerol were added to a final concentration of 1 M and 50 vol%, respectively, and the pH was adjusted to 8 with 1 M Tris/HCl, pH 7. The solution was incubated at 4 °C for 12 h, and dialysed against 50 mM Tris/HCl/1 M NaCl, pH 8, overnight and then against 50 mM Tris/HCl/0.1 M NaCl, pH 8, for 12 h.

### Purification of anhydrothrombin by benzamidine–Sepharose chromatography

All steps were carried out at 4 °C. The glycerol-free anhydrothrombin preparation was applied to a column of benzamidine–Sepharose (26 mm × 250 mm), equilibrated with 50 mM Tris/HCl/0.1 M NaCl, pH 7.5. The column was washed with the same buffer, and then anhydrothrombin was subsequently eluted with 0.2 M benzamidine in 50 mM Tris/HCl/0.1 M NaCl, pH 7.5. The eluate was dialysed extensively against 50 mM Tris/HCl/0.1 M NaCl, pH 6.5, to remove benzamidine, and *p*-nitrophenyl-*p*′-amidinophenyl methanesulphonate was then added at a final concentration of 1 mM to completely inactivate residual enzymic activity.

### Immobilization of anhydrothrombin on an agarose gel

Anhydrothrombin (27 mg), dissolved in 5 ml of 0.2 M $NaHCO_3$/0.5 M NaCl, pH 8.2, was applied to a Hi-trap NHS-activated column (16 mm × 25 mm). The column was incubated at 20 °C for 60 min without flow, and then washed with 0.5 M Tris/HCl/1 M NaCl, pH 8, for 3 h at a flow rate of 8.3 cm/h. Approx. 15 mg of anhydrothrombin bound to the column, as determined from the amount of unbound protein.

### SDS/PAGE

SDS/PAGE was performed under non-reducing conditions by using 4–20% gradient gels. Gels were stained with Coomassie Brilliant Blue R250. Molecular-mass marker Daiichi III was used as a molecular-mass standard.

### Affinity chromatography using anhydrothrombin–agarose

Each protein (1 mg; fibrinogen, factor XIII, protein C or antithrombin), dissolved in 50 mM Tris/HCl/0.1 M NaCl, pH 7.5, or 700 units of factor VIII dissolved in 50 mM Tris/HCl/0.1 M NaCl/10 mM $CaCl_2$, pH 7.5, was applied to an anhydrothrombin–agarose column equilibrated with the same buffer. The column was washed with the equilibration buffer until unbound proteins were completely removed. The elution was performed at a flow rate of 0.2 ml/min by two different methods: one method used an eluent consisting of 0.15 M benzamidine in 50 mM Tris/HCl/0.1 M NaCl, pH 7.5, and the other was with an NaCl gradient from 0.1 to 1 M, in 50 mM Tris/HCl, pH 7.5.

### Determination of binding constants using an IAsys resonant mirror biosensor

Kinetic parameters were determined by using an IAsys single-channel resonant mirror biosensor (Affinity Sensors, Cambridge, U.K.) [28,29]. Anhydrothrombin was covalently bound to an activated carboxymethyl-dextran cuvette using a protocol provided by the manufacturer. According to the data provided by the manufacturer, 1000 arc seconds are defined as coupling of approx. 5 ng of protein/mm$^2$ of sensing surface. To determine kinetic parameters, binding assays for each substrate were carried out at four different protein concentrations. The interactions of fibrinogen, factor XIII and protein C with the immobilized anhydrothrombin were monitored in 10 mM phosphate/2.7 mM KCl/137 mM NaCl/0.05% Tween 20, pH 7.4, and binding of factor VIII was monitored in 50 mM Tris/HCl/2.7 mM KCl/137 mM NaCl/5 mM $CaCl_2$/0.05% Tween 20, pH 7.4. The cuvette surface was regenerated by washing with a solution of 2 M NaCl/0.2 M benzamidine. Interactions of fibrinogen with the slow and fast forms of anhydrothrombin were monitored in 50 mM Tris/HCl/0.15 M choline chloride (ChCl), pH 7.4, and in 50 mM Tris/HCl/0.15 M NaCl, pH 7.4, respectively. Interactions of factor VIII with slow- and fast-form anhydrothrombin were monitored in 50 mM Tris/HCl/0.15 M ChCl/5 mM $CaCl_2$, pH 7.4, and in 50 mM Tris/HCl/0.15 M NaCl/5 mM $CaCl_2$, pH 7.4. Binding was measured at 2 s intervals, and the data readout from the biosensor was measured in units of arc seconds. All binding experiments were performed in duplicate for factor VIII and several times for factor XIII, fibrinogen and protein C at 25 °C. Binding parameters were calculated from the association and dissociation phases of the binding reactions by using the non-linear curve-fitting Fast Fit software [30] provided by the manufacturer (Affinity Sensors).

## RESULTS

### Preparation of anhydrothrombin

After repeated treatment of thrombin with PMSF, the amidolytic activity of PMS-thrombin preparation was reduced to 0.8%. Alkaline treatment of PMS-thrombin to eliminate PMS was carried out according to Ako et al. [19]. The anhydro reaction was monitored by measuring the dehydroalanine content [27]. To obtain a completely refolded anhydrothrombin preparation, it was essential to perform dialysis in two steps, inasmuch as when the second dialysis was carried out directly without the first dialysis in the presence of 50% glycerol, virtually all proteins aggregated and precipitated. The recovery of anhydrothrombin after the second dialysis step was 49 mg (82% yield) from 60 mg of thrombin. The final preparation of anhydrothrombin after benzamidine–Sepharose chromatography, followed by treatment with *p*-nitrophenyl-*p*′-amidinophenyl-methanesulphonate, retained no detectable amidase activity. The final yield of the preparation of anhydrothrombin was 31 mg (52%).

### SDS/PAGE of anhydrothrombin

SDS/PAGE of anhydrothrombin is shown in Figure 1, together with that of thrombin, antithrombin, thrombin + antithrombin

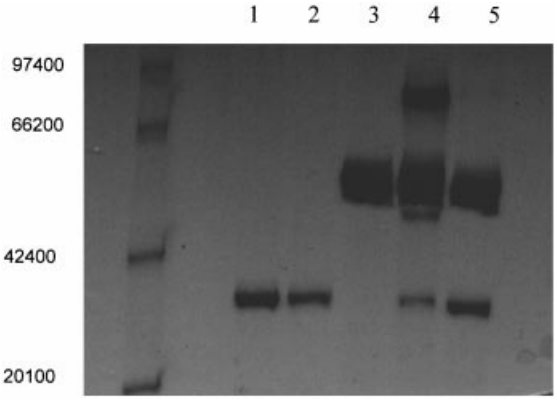


**Figure 1 SDS/PAGE of anhydrothrombin**

Lane 1, thrombin; lane 2, anhydrothrombin; lane 3, antithrombin; lane 4, thrombin + antithrombin; lane 5, anhydrothrombin + antithrombin. Samples for lanes 4 and 5 were incubated for 30 min at 37 °C prior to SDS treatment.

**Table 1 Kinetic parameters for binding of thrombin substrates to immobilized anhydrothrombin**

| Thrombin substrate | $k_{ass}$ ($M^{-1} \cdot s^{-1}$) | $k_{diss}$ ($s^{-1}$) | $K_d$ (M) |
|---|---|---|---|
| Factor VIII | $1.1 \times 10^{6}$ | $1.2 \times 10^{-2}$ | $1.2 \times 10^{-8}$ |
| Fibrinogen | $2.8 \times 10^{5}$ | $1.2 \times 10^{-2}$ | $4.4 \times 10^{-8}$ |
| Factor XIII | $3.6 \times 10^{4}$ | $8.6 \times 10^{-3}$ | $2.8 \times 10^{-7}$ |
| Protein C | $0.92 \times 10^{2}$ | $7.4 \times 10^{-3}$ | $8.1 \times 10^{-5}$ |

and anhydrothrombin + antithrombin. Samples on lanes 4 and 5 (Figure 1) were incubated for 1 h at 37 °C prior to SDS/PAGE. Compared with lane 4, which exhibits a complex of thrombin and antithrombin (78000 Da), there is no comparable band in lane 5, suggesting that anhydrothrombin could not form a covalently bonded complex with antithrombin.

### Affinity chromatography using anhydrothrombin–agarose

Fibrinogen, factor VIII, factor XIII and protein C readily bound to an anhydrothrombin–agarose column, and were eluted with 0.2 M benzamidine in 50 mM Tris/HCl containing 0.1 M NaCl, pH 7.5. When the elution was carried out with a linear NaCl gradient (0.1–1.5 M), factor XIII was partially eluted, and fibrinogen and factor VIII were eluted as a broad peak at a concentration of about 0.8 M NaCl. Protein C was eluted at a concentration of 1 M NaCl, and it could also be eluted by 0.1 M $CaCl_2$. Antithrombin was bound very weakly to the anhydrothrombin–agarose column and eluted with the wash buffer.

### Kinetic parameters for interactions of various thrombin substrates with anhydrothrombin

A biosensor response of approx. 2000 arc seconds for anhydrothrombin was observed during the coupling reaction. According to the data provided by the manufacturer, 10 ng of anhydrothrombin/$mm^2$ of the sensor surface was estimated to have been bound. Data obtained with the biosensor were transferred directly to the Fast Fit program provided by the manufacturer. The kinetic parameters estimated for the interaction of various thrombin substrates with anhydrothrombin are summarized in Table 1. The typical interaction patterns of fibrinogen and factor VIII with the fast and slow forms of anhydrothrombin are shown in Figures 2 and 3, respectively. Kinetic parameters for interactions of fibrinogen with the fast form of anhydrothrombin were estimated as $k_{ass} = 1.6 \times 10^5$ $M^{-1} \cdot s^{-1}$, $k_{diss} = 6.9 \times 10^{-3}$ $s^{-1}$ and $K_d = 4.3 \times 10^{-8}$ M, and those with the slow form of anhydrothrombin were $k_{ass} = 6.1 \times 10^5$ $M^{-1} \cdot s^{-1}$, $k_{diss} = 6.9 \times 10^{-3}$ $s^{-1}$ and $K_d = 4.4 \times 10^{-8}$ M. Kinetic

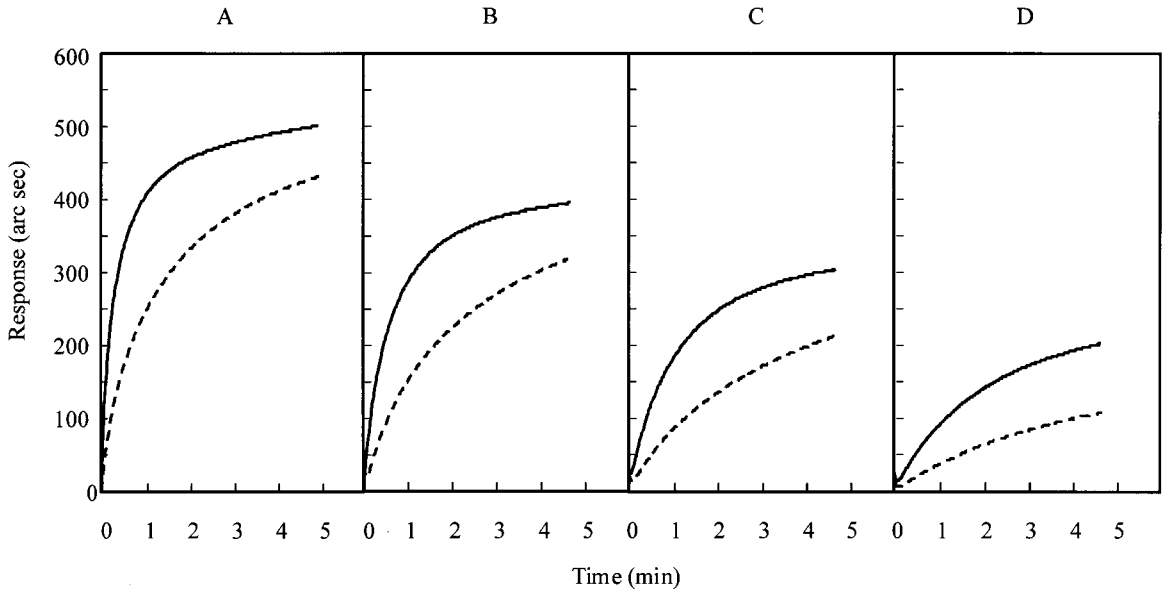


**Figure 2 Interaction of the fast and slow forms of anhydrothrombin with fibrinogen**

Fibrinogen was incubated at different concentrations in an anhydrothrombin-immobilized cuvette in the presence of 0.15 M NaCl (solid lines) or 0.15 M ChCl (broken lines). Fibrinogen concentrations: (**A**) $1.57 \times 10^{-7}$ M; (**B**) $7.85 \times 10^{-8}$ M; (**C**) $3.92 \times 10^{-8}$ M; (**D**) $1.96 \times 10^{-8}$ M.

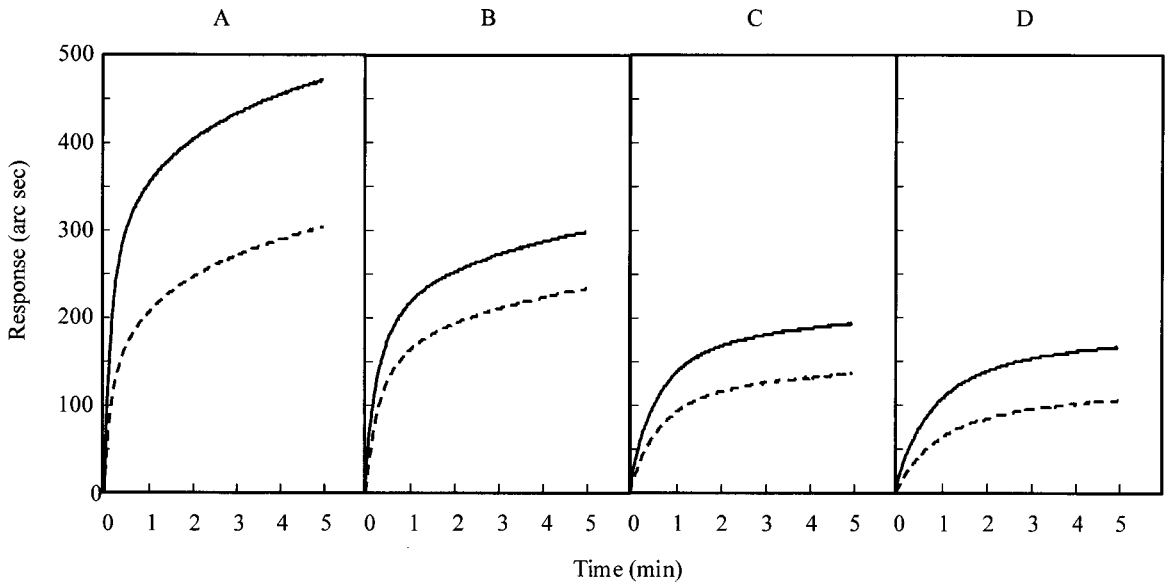


**Figure 3 Interaction of the fast and slow forms of anhydrothrombin with factor VIII**

Factor VIII was incubated at different concentrations in an anhydrothrombin-immobilized cuvette in the presence of 0.15 M NaCl (solid lines) or 0.15 M ChCl (broken lines). Factor VIII concentrations: (**A**) $5.97 \times 10^{-8}$ M; (**B**) $2.98 \times 10^{-8}$ M; (**C**) $1.49 \times 10^{-8}$ M; (**D**) $9.94 \times 10^{-9}$ M.

parameters for interactions of factor VIII with the fast form of anhydrothrombin were estimated as $k_{ass} = 1.0 \times 10^6$ $M^{-1} \cdot s^{-1}$, $k_{diss} = 9.4 \times 10^{-3}$ $s^{-1}$ and $K_d = 9.4 \times 10^{-9}$ M, and those with the slow form of anhydrothrombin were $k_{ass} = 5.1 \times 10^5$ $M^{-1} \cdot s^{-1}$, $k_{diss} = 1.4 \times 10^{-2}$ $s^{-1}$ and $K_d = 2.8 \times 10^{-8}$ M. Thus as anticipated, anhydrothrombin revealed higher affinity for both fibrinogen and factor VIII in the presence of NaCl (fast form) than in the presence of ChCl (slow form). Fibrinogen and protein C were dissociated from an anhydrothrombin-immobilized cuvette by 2 M NaCl, and factor VIII was dissociated by 0.2 M benzamidine. Factor XIII was dissociated only after a few washes with 2 M NaCl and 0.2 M benzamidine.

## DISCUSSION

The preparation of anhydrothrombin was initially reported by Tomono and Sawada [31]. These investigators incubated PMS-thrombin under conditions where thrombin does not denature (pH 9, 24 h, 4 °C), and reported that they could purify antithrombin by anhydrothrombin–Sepharose chromatography [31]. Bauer et al. [32] reported that guanidium hydrochloride (GdnHCl)- or urea-denatured thrombin could be properly refolded by diluting the protein solution to non-denaturing concentrations of these reagents. Ashton and Scheraga [33] synthesized anhydrothrombin using 6 M GdnHCl for solubilization purposes, and subsequently refolded the denatured anhydrothrombin by a quick 10-fold dilution of the protein in GdnHCl. When we attempted to prepare the anhydrothrombin under the conditions of pH 9 at 4 °C for 24 h, PMS elimination failed to occur and the derivative did not bind to benzamidine–Sepharose, similar to that reported by Ashton and Scheraga [33]. In the benzamidine–Sepharose chromatography process, Ashton and Scheraga washed the column with a solution of low ionic strength, since 6 M GdnHCl is so deleterious to thrombin that anhydrothrombin failed to refold completely and bound only weakly to benzamidine–Sepharose. Accordingly, we attempted to synthesize anhydrothrombin under milder conditions. The elimination reaction to prepare anhydrothrombin, as well as anhydrochymotrypsin and anhydrotrypsin, required an incubation of the PMSF-inactivated protease at a pH > 11. In contrast with anhydrotrypsin and anhydrochymotrypsin, virtually all the anhydrothrombin aggregated in the refolding process at neutral pH. However, anhydrothrombin did not aggregate in alkaline solution, possibly because of its overall negative charge and molecules repelling one another. Aggregation of anhydrothrombin at neutral pH was successfully prevented by the addition of glycerol to the refolding solution, and the refolded anhydrothrombin could be recovered upon removal of glycerol by dialysis. When we synthesized anhydrothrombin at a higher protein concentration in the refolding process, the recovery decreased to less than 50 %, mainly due to aggregation.

Each protein substrate of thrombin bound to an anhydrothrombin–agarose column. We determined their kinetic parameters with anhydrothrombin by IAsys. Fibrinogen and factor VIII are both thought to interact with the exosite I of thrombin, and revealed high affinity for anhydrothrombin. Interestingly, factor VIII had a higher affinity than fibrinogen and factor XIII for anhydrothrombin under physiological conditions. The interactions of anhydrothrombin with fibrinogen and factor VIII varied in the presence and absence of $Na^+$. Thus the $Na^+$ binding to anhydrothrombin enhanced the association rate ($k_{ass}$) by approx. 2.5- and 2-fold for fibrinogen and factor VIII, respectively, which suggests that the transformation of thrombin between the fast ($Na^+$-bound) and slow ($Na^+$-free) forms may regulate the interactions of thrombin with fibrinogen and factor VIII. In contrast, protein C and antithrombin had rather low affinity for anhydrothrombin. These results may be interpreted as meaning that they need cofactors, i.e. thrombomodulin for protein C and heparin for antithrombin, to interact with thrombin *in vivo*, or that they do not interact with the exosite I of thrombin.

By using glycerol in a refolding process, it became possible to obtain anhydrothrombin in high yield without aggregation under

milder conditions. In fact, using this refolding method, we could prepared anhydro-factors VIIa and Xa, and activated protein C (results not shown), all of which have higher molecular masses than thrombin.

Anhydrothrombin and other anhydro-serine proteinases may have a wide variety of applications. First, anhydrothrombin can be used in affinity chromatography to purify thrombin substrates, among which factor VIII, factor XIII and protein C have already been utilized as therapeutics and required in large quantities with high purity. Studies on the application of other anhydro-serine proteinases are also in progress.

Secondly, they are useful for analysis of detailed interactions between serine proteases and their substrates. It has been impossible to analyse these interactions directly because of their catalytic activities, and anhydro-serine proteinases make these studies possible. For example, the binding sites of factor VIII for factor Xa and thrombin were determined by Nogami et al. using anhydro-factor Xa [34] and anhydrothrombin [35], respectively. We conclude that anhydrothrombin and other anhydro-serine proteinases may be useful tools to purify their substrates as well as to study their interactions with substrate proteins in detail.

## REFERENCES


1 Lorand, L. and Konishi, K. (1964) Activation of the fibrin stabilizing factor of plasma by thrombin. Arch. Biochem. Biophys. **105**, 58–67

2 Colman, R. W. (1969) The effect of proteolytic enzymes on bovine factor V. Biochemistry **8**, 1438–1444

3 Fass, D. N., Knutson, G. J. and Katzmann, J. D. (1982) Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. Blood **59**, 594–600

4 Andersen, H., Greenberg, D. L., Fujikawa, K., Xu, W., Chung, D. W. and Davie, E. W. (1999) Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc. Natl. Acad. Sci. U.S.A. **96**, 11189–11193

5 Vu, T.-K. H., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell **64**, 1057–1068

6 Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Moff, S., Farese, Jr, R. V., Tam, C. and Coughlin, S. R. (1998) A dual thrombin receptor system for platelet activation. Nature (London) **394**, 690–694

7 Esmon, N. L., Owen, W. G. and Esmon, C. T. (1982) Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem. **257**, 859–864

8 Orthner, C. L. and Kosow, D. P. (1980) Evidence that human $\alpha$-thrombin is a monovalent cation-activated enzyme. Arch. Biochem. Biophys. **202**, 63–75

9 Wells, C. M. and Di Cera, E. D. (1992) Thrombin is a $Na^+$-activated enzyme. Biochemistry **31**, 11721–11730

10 Dang, D. Q., Vindigni, A. and Di Cera, E. (1995) An allosteric switch controls the procoagulant and anticoagulant activities of thrombin. Proc. Natl. Acad. Sci. U.S.A. **92**, 5977–5981

11 Di Cera, E., Guito, R. E., Vindigni, A., Dang, D. Q., Ayala, M. Y., Wuyi, M. and Tulinsky, A. (1995) The $Na^+$ binding site of thrombin. J. Biol. Chem. **270**, 22089–22092

12 Hagen, T. C., Endres, G. F. and Scheraga, H. A. (1975) Mechanism of action of thrombin on fibrinogen. Arch. Biochem. Biophys. **171**, 327–336

13 Rydel, T. J., Tulinsky, A. and Bode, W. (1991) Refined structure of the hirudin-thrombin complex. J. Mol. Biol. **221**, 583–601

14 Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hosfteenge, J. (1989) The refined 1.9 Å crystal structure of human $\alpha$-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J. **8**, 3467–3475

15 Stubbs, M. T. and Bode, W. (1995) The clot thickens: clues provided by thrombin structure. Trends Biochem. Sci. **20**, 23–28.

16 Myles, T., Church, F. C., Whinna, H. C., Monard, D. and Stone, S. R. (1998) Role of thrombin anion-binding exosite-I in the formation of thrombin-serpin complex. J. Biol. Chem. **273**, 31203–31209

17 Dharmawardana, K. R., Olson, S. T. and Bock, P. E. (1999) Role of regulatory exosite I in binding of thrombin to human factor V, factor Va, factor Va subunits, and activation fragment. J. Biol. Chem. **274**, 18635–18643

18 Ako, H., Ryan, C. A. and Foster, R. J. (1972) The purification by affinity chromatography of a proteinase inhibitor binding species of anhydro-trypsin. Biochem. Biophys. Res. Commun. **46**, 1639–1645

19 Ako, H., Foster, R. J. and Ryan, C. A. (1972) The preparation of anhydro-trypsin and its reactivity with naturally occurring proteinase inhibitor. Biochem. Biophys. Res. Commun. **47**, 1402–1407

20 Ako, H., Foster, R. J. and Ryan, C. A. (1974) Mechanism of action of naturally occurring proteinase inhibitors. Studies with anhydrotrypsin and anhydrochymotrypsin purified by affinity chromatography. Biochemistry **13**, 132–139

21 Yokosawa, H. and Ishii, S. (1977) Anhydrotrypsin: new features in ligand interactions revealed by affinity chromatography and thionine replacement. J. Biochem. (Tokyo) **81**, 647–656

22 Yokosawa, H., Ojima, S. and Ishii, S. (1977) Chemical transformation of the active-site serine residue of *Streptomyces griseus* trypsin to a cysteine residue. J. Biochem. (Tokyo) **82**, 869–876

23 Fenton, J. W., Fasco, M. J. and Stackrow, A. B. (1977) Human thrombins. J. Biol. Chem. **252**, 3587–3598

24 Shima, M., Nakai, H., Scandella, D., Tanaka, I., Sawamoto, Y., Kamisue, S., Morichika, S., Murakami, T. and Yoshioka, A. (1995) Common inhibitory effects of human anti-C2 domain inhibitor alloantibodies on factor VIII binding to von Willebrand factor. Br. J. Haematol. **91**, 714–721

25 Matsuda, M., Iwanaga, S. and Nakamura, S. (1972) A simple, large scale method for preparation of plasminogen-free fibrinogen. Thromb. Res. **1**, 619–630

26 Koide, T., Odani, S. and Ono, T. (1985) Human histidine-rich glycoprotein: simultaneous purification with antithrombin III and characterization of its gross structure. J. Biochem. (Tokyo) **98**, 1191–1200

27 Weiner, H., White, M. N., Hoare, D. G. and Koshland, Jr, D. E. (1966) The formation of anhydro-chymotrypsin by removing the elements of water from the serine at active site. J. Am. Chem. Soc. **88**, 3851–3859

28 Cush, R., Cronin, J. M., Stewart, W. J., Maule, C. H., Molloy, J. and Goddard, N. J. (1993) The resonant mirror: a novel optical biosensor for direct sensing of biomolecular interactions. Part I: Principle of operation and associated instrumentation. Biosens. Bioelectr. **8**, 347–354

29 Buckle, P. E., Davies, R. J., Kinning, T., Yeung, D., Edwards, P. R., Pollard-Knight, D. and Lowe, C. R. (1993) The resonant mirror: a novel optical biosensor for direct sensing of biomolecular interactions. Part II: Application. Biosens. Bioelectr. **8**, 355–363

30 George, A. J. T., French, R. R. and Glennie, M. J. (1995) Measurement of kinetic binding constants of a panel of anti-saporin antibodies using a resonant mirror biosensor. J. Immunol. Methods **183**, 51–63

31 Tomono, T. and Sawada, E. (1986) Preparation of anhydro-thrombin and its interaction with plasma antithrombin III. Acta Haematol. Jpn **49**, 969–979

32 Bauer, R. S., Chang, T. L. and Berliner, L. J. (1980) Stability differences between high coagulant ($\alpha$) and noncoagulant ($\gamma$) human thrombins. J. Biol. Chem. **255**, 5900–5903

33 Ashton, R. W. and Scheraga, H. A. (1995) Preparation and characterization of anhydrothrombin. Biochemistry **34**, 6454–6463

34 Nogami, K., Shima, M., Hosokawa, K., Suzuki, T., Koide, T., Saenko, L. E., Scandella, D., Shibata, M., Kamisue, S., Tanaka, I. and Yoshioka, A. (1999) Role of factor VIII C2 domain in factor VIII binding to factor Xa. J. Biol. Chem. **274**, 31000–31007

35 Nogami, K., Shima, M., Hosokawa, K., Suzuki, T., Koide, T., Saenko, L. E., Tanaka, I., Shibata, M. and Yoshioka, A. (2000) The factor VIII C2 domain contains the thrombin binding site responsible for thrombin-catalyzed cleavage at $Arg^{1689}$. J. Biol. Chem. **275**, 25774–25780


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