J Biochem 130, 269–277 (2001)

An Efficient Refolding Method for the Preparation of Recombinant Human Prethrombin-2 and Characterization of the Recombinant-Derived α-Thrombin

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Received April 12, 2001, accepted May 23, 2001

Human recombinant prethrombin-2 was produced in *Escherichia coli*. The expressed prethrombin-2 formed intracellular inclusion bodies from which the protein was refolded by a simple one-step dilution process in buffer consisting of 50 mM Tris-HCl, containing 20 mM $CaCl_2$, 500 mM NaCl, 1 mM EDTA, 600 mM arginine, 1 mM cysteine, 0.1 mM cystine, 10% (v/v) glycerol, and 0.2% (w/v) Brij-58 at pH 8.5. After refolding, prethrombin-2 was purified by hirudin-based COOH-terminal peptide affinity chromatography, and then activated with *Echis carinatus* snake venom prothrombin activator (ecarin). The activated protein, α-thrombin, was then tested for several activities including activity toward chromogenic substrate, release of fibrinopeptide A from fibrinogen, activation of protein C, and thrombin-activatable fibrinolysis inhibitor, reactivity with antithrombin, clotting activity, and platelet aggregation. The kinetic data showed no differences in activity between our recombinant α-thrombin and plasma-derived α-thrombin. The yield of refolded recombinant human prethrombin-2 was about 4–7% of the starting amount of solubilized protein. In addition, the final yield of purified refolded protein was 0.5–1%, and about 1 mg of recombinant prethrombin-2 could be isolated from 1 liter of *E. coli* cell culture.

Key words: α-thrombin, inclusion body, prethrombin-2, recombinant, refolding.

Thrombin, which plays a central role in hemostasis, is a multifunctional serine protease belonging to the trypsin family (*1–3*). Thrombin has a variety of biochemical properties and interactions, acting as both a procoagulant and anticoagulant. The coagulation of blood is achieved through a complex series of reactions resulting in the formation of a fibrin clot. Prothrombin is activated to the active protease, thrombin, in the final step of the coagulation cascade. Thrombin is responsible for the proteolytic removal of fibrinopeptides A and B from fibrinogen, which results in the formation of a fibrin clot. On the other hand, in the presence of thrombomodulin, thrombin efficiently activates protein C. Activated protein C degrades coagulation factors Va and VIIIa, and thereby inhibits blood coagulation (*4*).

Human prothrombin, the inactive precursor of thrombin, is a single-chain glycoprotein with a total of 579 amino acid residues. Prothrombin contains a Gla domain, two kringle domains, and a protease domain, and has three carbohydrate chains that are *N*-linked at the 78th, 100th, and 373rd Asn residues. This molecule, a serine protease zymogen with a molecular weight of 72,000, is synthesized in the liver and secreted into the blood. As the blood coagulation cascade progresses, prothrombin undergoes limited proteolysis at the Arg271-Thr272 and Arg320-Ile321 bonds by prothrombinase (a complex of factor Xa, factor Va, calcium, and phospholipids) to form two polypeptide chains, A and B, linked by a single disulfide bond, α-thrombin, with 308 amino acid residues.

Prethrombin-2, the smallest single-chain immediate precursor of α-thrombin (corresponding to residues Thr272 to Glu579 in prothrombin), has one glycosylation site at Asn373 and four disulfide bonds, Cys293-Cys439, Cys348-Cys364, Cys493-Cys507, and Cys521-Cys551 (*4*, *5*).

The present study was performed to develop an expression system for pathogen-free recombinant thrombin, which could be used as a pharmaceutical reagent, for example, as an aid in hemostasis or one component of a fibrin sealant. The most widely used and simplest expression systems are those using *E. coli* as the host. Therefore, we used an *E. coli* expression system and chose prethrombin-2, the smallest precursor of α-thrombin, because of its ease of expression in *E. coli*. However, as is often the case, this normally soluble eukaryotic protein was found to be insoluble in *E. coli*, yielding inclusion bodies from which the protein had to be solubilized and refolded

There have been reports of the overproduction of human prethrombin-2 in *E. coli* (*6*, *7*), but little physiologically

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Abbreviations Gla, γ-carboxyglutamic acid, FPA, fibrinopeptide A, GAG-UTM, recombinant glycosaminoglycan (GAG)-modified urinary thrombomodulin; TAFI, thrombin-activatable fibrinolysis inhibitor, TAFIa, activated thrombin-activatable fibrinolysis inhibitor; AT III, antithrombin; APC, activated protein C, IPTG, *iso*propyl-1-thio-β-D-galactopyranoside, F-FPR-ck, fluorescein-Phe-Pro-Arg-chloromethyl ketone, *p*NA, *p*-nitroanilide; PEG, polyethylene glycol

active human thrombin has been prepared. On the other hand, reversible sulfonation of cysteines in bovine prethrombin-2 has been reported to break any incorrect disulfide pairing and to stabilize the unfolded protein (*8*). However, there have been no previous reports of a simple and efficient refolding method for the production of human recombinant prethrombin-2 that would make it possible to characterize the protein molecule. We describe here an efficient refolding method for the preparation of human recombinant prethrombin-2, and also the characterization of recombinant α-thrombin.

MATERIALS AND METHODS

Materials—Human plasma-derived α-thrombin (3,290 NIH units/mg), human antithrombin (AT III) (specific activity, 0.8 mol of thrombin/mol of AT III), and fluorescein-Phe-Pro-Arg-chloromethyl ketone (F-FPR-ck) were from Haematologic Technologies (Vermont, USA); human fibrinogen (plasminogen and von Willebrand factor–depleted, and more than 95% clottable) and human thrombin-activatable fibrinolysis inhibitor (TAFI) were from Enzyme Research Laboratories (Indiana, USA), S-2238 (H-D-Phe-Pip-Arg-*p*NA·2HCl) and S-2366 (*pyro*-Glu-Pro-Arg-*p*NA·HCl) were from Chromogenix (Stockholm, Sweden); 3-(2-furyl) acryloyl-Ala-Arg-OH was from Bachem (Bubendorf, Switzerland); human factor II–deficient plasma was from George King Bio-Chemical (Kansas, USA); hirudin (leech, recombinant, Lys47-rHV2 variant), polyethyleneglycol (PEG) 3350 and 8000, human serum albumin (fatty acid free), 2,2,2-trifluoroethanol, and ecarin were from Sigma-Aldrich; lysozyme (egg white) was from Seikagaku (Tokyo); recombinant *N*-glycosidase F was from Roche Diagnostics, benzamidine-HCl was from Tokyo Chemical Industries; Brij-35 and Brij-58 were from Nacalai Tesque (Kyoto). All other chemicals were of analytical grade or the highest quality commercially available and were obtained from Wako Pure Chemical Industries (Osaka). Recombinant glycosaminoglycan (GAG)-modified urinary thrombomodulin (GAG-UTM) was a generous gift from Drs. T. Edano and K. Onoki, Kowa (Tokyo) (*9*, *10*). Human protein C and activated protein C were prepared as previously reported (*11*) Restriction enzymes and other enzymes for DNA sequencing and cloning were purchased from New England Biolabs (Massachusetts, USA), and Takara Shuzo (Shiga). Expression vector plasmids pKK233-2 and pUC18 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden) and Takara Shuzo, respectively. *E. coli* strain JM109 (Toyobo, Osaka) was used for cloning and expression.

Plasmid Constructs—Prothrombin cDNA was synthesized from mRNA extracted from liver (Sawady Technology, Tokyo) with a T-Primed First Strand Kit (Amersham Pharmacia Biotech AB). Sequences of the specific primers used to clone the prothrombin cDNA were as follows: 5′-ATGGCGCACGTCCGAGGCTTGCAGCTGCCT-3′ (forward primer, named PT1), and 5′-CTACTCTCCAAACTGATCAATGACCTTCT-3′ (reverse primer, named PT2). The cDNA was cloned into pCR2.1 (Invitrogen, California, USA) and sequenced. The deduced amino acid sequence was identified as a known prothrombin sequence (*12*). To construct an *E. coli* prethrombin-2 expression plasmid, the coding region was amplified by PCR with a forward primer PT3 (5′-ATGCCCATGGCCACAAGTGAGTAC-3′) corresponding to the 5′-*Nco*I site, and a reverse primer PT4 (5′-TAGCATAAGCTTCTACTCTCCAAACTGATCAAT-3′) corresponding to the 3′-*Hin*dIII site. To construct the high-level expression plasmid vector pUT1, the origin of replication on pKK233-2 was replaced with that of pUC18. An *Eco*RI linker was inserted into the *Pvu*I site of pUC18. The *Eco*RI/*Pvu*I fragment from pUC18 was inserted into the *Eco*RI/*Pvu*I sites of pKK233-2. The prethrombin-2 DNA fragment digested with *NcoI*/*Hin*dIII was subcloned into pUT1 digested with *NcoI*/*Hin*dIII (Fig. 1). The vector was confirmed to contain the entire sequence of the human prethrombin-2 DNA fragment by sequencing on an automated DNA sequencer.

Cultivation and Harvesting of E. coli—The host *E. coli* strain transformed with plasmid containing the *trc* promoter and the gene for human prethrombin-2 was grown at 30°C in 200 ml of LB medium containing 50 μg/ml ampicillin. The overnight cultures were transferred into 8 liters of LB medium in a fermenter and incubated until they reached an OD_{600} of 0.2 to 0.5 at 30°C. Expression from the *trc* promoter was induced by adding *iso*propyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM and cultured overnight at 30°C. The cells were harvested by centrifugation for 30 min at 4°C.

Purification and Extraction of Inclusion Bodies—The cell pellet was resuspended in distilled water. The cells were lysed by adding lysozyme to a final concentration of 0 6 mg/ml and incubating at room temperature for 30 min followed by digestion overnight at 4°C. This suspension was sonicated and centrifuged for 20 min at 4°C.

The pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 1% Triton X-100, and the suspension was sonicated for 1 min with a 50% duty cycle (*i.e.* on for 0.5 s, then off for 0.5 s), and centrifuged for 20 min at 4°C. This process was repeated several times, and then repeated several times using distilled water. The clarified inclusion body extract was stored at −20°C until use.

Solubilization and Refolding of Prethrombin-2 from Inclusion Bodies—The inclusion body pellet was resuspended in 23 mM ammonium acetate containing 6 M guanidine hydrochloride, 10 mM EDTA, and 30 mM cysteine, pH 9.5, to a final concentration of 10 mg/ml (containing about 2 mg/ml of protein). The sample was then sonicated for a few minutes with a 50% duty cycle in an ice bath and allowed to stand for 2 h at room temperature. The suspension was centrifuged for 10 min at 10,000 ×*g*, and the supernatant was filtrated through a 0 45 μm membrane filter. The solubilized protein concentration was determined using bovine serum albumin as a standard with protein assay reagent (Bio-Rad Laboratories, California), based on the method reported by Bradford (*13*). Folding was initiated by diluting the solubilized protein into various refolding buffers, as shown in Table I, to give a final protein concentration of 50–100 μg/ml (corresponding to about 20 to 40-fold dilution) at room temperature followed by incubation overnight at room temperature.

Estimation of Refolding Efficiency—The extent of prethrombin-2 refolding was assessed by the S-2238 hydrolysis assay. Aliquots of the refolding solution were dialyzed against 50 mM Tris-HCl, 100 mM NaCl, pH 8.0, overnight at 4°C. Then, 50 μl of dialyzed prethrombin-2 was activated to α-thrombin by adding 50 μl of ecarin (2 units/ml) and incubating at 37°C for 2 h. One hundred microliters of the

chromogenic substrate S-2238 (2 mM) was then added to the above α-thrombin solution, and the α-thrombin activity was monitored by the absorbance change at 405 nm (SpectraMAX, Molecular Devices, California) at 37°C. The amounts of α-thrombin were calculated by reference to a standard curve constructed using known amounts of plasma-derived α-thrombin. The refolding efficiency (%) defined in this report was as follows

Refolding efficiency (%) = amount of α-thrombin (determined by S-2238 hydrolysis assay)/amount of solubilized protein (determined with protein assay reagent) × 100 (%).

Large Scale Preparation and Purification of Refolded Prethrombin-2—About 600 mg of the inclusion body pellet was resuspended and solubilized as described above. The solubilized protein was diluted into pH 8.5 buffer consisting of 50 mM Tris-HCl, 20 mM $CaCl_2$, 500 mM NaCl, 1 mM EDTA, 600 mM arginine, 1 mM cysteine, 0.1 mM cystine, 10% (v/v) glycerol, and 0.2% (w/v) Brij-58. About 2 liters of the refolding solution was dialyzed for 1–2 days against 20 mM citrate buffer, pH 5.5, at 4°C. The precipitated proteins were removed by filtration through a 0 45 μm membrane. The pass-through fraction was loaded onto an SP-Sepharose column (2.6 × 10 cm), equilibrated with 20 mM citrate buffer, pH 5.5, and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM citrate buffer, pH 5.5. The eluted protein was further subjected to affinity chromatography (0.5 × 5 cm) using an immobilized hirudin-based COOH-terminal peptide (NH_2-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-COOH), and eluted with a linear gradient of NaCl. This affinity column was prepared by a modification of the procedure of Fisher *et al.* (*14*). Purified prethrombin-2 was dialyzed against 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, overnight at 4°C and stored at −80°C until use.

Activation of Refolded Prethrombin-2—About 1 mg of purified prethrombin-2 was activated using ecarin as follows. Aliquots of 20 μg/ml of purified protein were incubated for 20 h with 2 units/ml ecarin in 50 mM Tris-HCl, 0.1% PEG 8000, 50 mM benzamidine-HCl, and 100 mM NaCl, pH 8.0, at room temperature. The reaction mixture was subjected to hirudin-based COOH terminal peptide affinity chromatography (0.5 × 5 cm) as described above. After loading, the column was washed with 0.5 M NaCl to remove the prethrombin-2 not activated by ecarin, and then the activated thrombin was eluted with 1.2 M KSCN. Finally, the eluted thrombin was dialyzed against 50 mM Tris-HCl containing 100 mM NaCl, pH 8.0, overnight at 4°C.

SDS-PAGE—SDS-PAGE was performed according to the method of Laemmli (*15*). The gel was stained with Coomassie Brilliant Blue R-250.

NH_2-Terminal Sequencing—Automated sequence analysis was performed with a PE-Applied Biosystems 492 protein sequencer.

Active Site Titration—Human plasma-derived α-thrombin was titrated with *p*-nitrophenyl *p'*-guanidinobenzoate as previously reported by Chase and Shaw (*16*). The active site titration of refolded recombinant α-thrombin was performed by calculating the ratio of the concentrations of fluorescein and the protein in the fluorescein-labeled α-thrombin using the above titrated plasma-derived α-thrombin as a standard. F-FPR-ck and α-thrombin were mixed (molar ratio of 4:1) and incubated for 2 h until α-thrombin activity toward S-2238 was no longer detectable. Free fluorescein inhibitor was removed by Micro Bio-Spin gel filtration (Bio-Rad) The concentrations of fluorescein and protein in the complex were determined by spectrofluorometry and the Bradford method, respectively. Fluorescein-labeled α-thrombin was excited at 485 nm and emission was detected at 535 nm in 96-well microwell plates with a microplate spectrofluorometer (SPECTRAFLUOR, TECAN Austria GmbH).

Steady-State Kinetics of S-2238 Hydrolysis—The initial rates of S-2238 hydrolysis, monitored by the absorbance change at 405 nm (SpectraMAX), were measured for the recombinant and plasma-derived α-thrombins using 0.5 nM α-thrombin with various concentrations of S-2238 ranging from 3 to 25 μM in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, and 100 mM NaCl, pH 8.0, at 37°C. K_m and k_{cat} were determined by fitting the data to the Hanes-Woolf plot (*17*).

Fibrinopeptide A Release Assay—The assay for thrombin-mediated fibrinopeptide A (FPA) release was performed by the method of Ng *et al.* (*18*). Briefly, reactions were performed at 37°C in 50 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 0.1% PEG 8000. The concentrations of α-thrombin and human fibrinogen used were 0.2 and 340 nM, respectively. The mixtures were incubated at 37°C for 0, 1, 2, 3, 4, 5, 7, 9, and 12 min, and 3 M perchloric acid was added to an aliquot of the reaction mixture to terminate the reaction at each time point (final concentration 0.3 M). All samples were filtered through a 0.45 μm membrane filter and analyzed by HPLC (Agilent Technologies, California) on a Protein & Peptide C18 reverse-phase HPLC column (Vydac, California) with acetonitrile gradient elution. All FPA data were fitted to a simple first-order equation. The values of k_{cat}/K_m were determined by dividing the first-order rate constants by the α-thrombin concentration as described (*18*).

Apparent Dissociation Constants for GAG-UTM—To estimate the apparent dissociation constants (K_{dapp}), various concentrations of GAG-UTM ranging from 0.16 to 2 μM in 10 μl aliquots were preincubated with 10 μl of 10 nM α-thrombin in buffer consisting of 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, 5 mM $CaCl_2$, and 100 mM NaCl, pH 7.5, in a total volume of 190 μl for 3 min at 37°C. To this mixture was then added 10 μl of 20 μM protein C to give a final volume of 200 μl (final concentrations as follows: GAG-UTM, ranging from 8 nM to 100 nM, 0.5 nM α-thrombin, and 1 μM protein C, respectively). After 90 s, the reaction was terminated by adding 1 μl of the specific α-thrombin inhibitor, hirudin (final concentration 5 units/ml). Then, to determine the amount of activated protein C generated, aliquots of 20 μl of each reaction mixture were diluted 8-fold with the above buffer, and kinetic analyses were carried out by adding 40 μl of 5 mM S-2366. The amount of activated protein C was calculated by reference to a standard curve constructed using known amounts of activated protein C. Standard activated protein C titrated using *p*-nitrophenyl *p'*-guanidinobenzoate as described above was used. K_{dapp} for GAG-UTM was calculated by fitting the data to the Hanes-Woolf plot.

Steady-State Kinetics of Protein C Activation—The activation of protein C was carried out in the absence or presence of 50 nM GAG-UTM. In the absence of GAG-UTM, 5 nM α-thrombin was incubated with various concentrations of protein C ranging from 1.25 to 40 μM in 50 mM Tris-

HCl, 0.1% PEG 8000, 0.01% human serum albumin, and 100 mM NaCl, pH 7.5, at 37°C for 15 min. In the presence of GAG-UTM, 0.5 nM α-thrombin was incubated with GAG-UTM and various concentrations of protein C ranging from 1.25 to 20 μM in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, 5 mM $CaCl_2$, and 100 mM NaCl, pH 7.5, at 37°C for 90 s. The reaction was terminated by adding the specific α-thrombin inhibitor, hirudin (final concentration 5 units/ml). Then, the amount of activated protein C was measured as described above.

Determination of k_{cat}/K_m for TAFI Activation—The assay of thrombin-mediated TAFI activation was carried out as follows. Reactions were performed at 22°C in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, 5 mM $CaCl_2$, and 100 mM NaCl, pH 7.8. The final concentrations of α-thrombin, GAG-UTM, and TAFI were 0.5 nM, 50 nM, and 100 nM, respectively. The mixtures were incubated for 0, 2, 4, 6, 8, 10, and 15 min, and then an aliquot of the reaction was terminated by the addition of hirudin to a final concentration of 5 units/ml. Then, quantification of activated TAFI (TAFIa) was carried out by adding 40 μl of 2.5 mM 3-(2-furyl) acryloyl-Ala-Arg-OH to 160 μl of each of the reaction mixtures. The TAFIa activity was determined from the initial rate of hydrolysis of the substrate 3-(2-furyl) acryloyl-Ala-Arg-OH by monitoring the absorbance change at 336 nm (*19*). The first-order rate constant was obtained from the slope by least-squares fitting to the straight line of a plot of $-\ln\{([TAFIa]_f-[TAFIa]_t)/([TAFIa]_f-[TAFIa]_0)\}$ *versus* time, where $[TAFIa]_f$, $[TAFIa]_0$, and $[TAFIa]_t$ are the final, baseline, and [TAFIa] values of each time point, respectively. The value after 120 min incubation was used as the final [TAFIa] value. The values of k_{cat}/K_m were determined by dividing the first-order rate constant by the α-thrombin concentration.

Thrombin Inactivation Rate by AT III—The assay was carried out under pseudo-first-order conditions in the absence of heparin. The inhibition reaction was initiated by adding α-thrombin (final concentration 5 nM) to various concentrations of AT III ranging from 0.1 to 1.7 μM in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, 5 mM $CaCl_2$, and 100 mM NaCl, pH 7.5, at 37°C, and sampled at appropriate time points to assay remaining α-thrombin activity by the rate of S-2238 hydrolysis. Inhibition by AT III was terminated by the addition of a large excess of S-2238 (final concentration 400 μM). The inhibition rates were estimated from the residual thrombin activities by measuring the initial rates of S-2238 hydrolysis as monitored by the absorbance change at 405 nm. The rates of inhibition of recombinant and plasma-derived α-thrombins by AT III without heparin were determined by substrate competition assay under pseudo-first-order conditions. The data were plotted as ln[active enzyme] against time, and from these slopes the pseudo-first-order rate constants were calculated. Then, the second-order rate constants were calculated from plots of the slopes of the pseudo-first-order rate constants against AT III concentration.

Clotting Activity Using Factor II–Deficient Human Plasma—Various concentrations of α-thrombin ranging from 6 to 18 nM in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, 5 mM $CaCl_2$, and 100 mM NaCl, pH 7.5, and factor II–deficient human plasma were preincubated at 37°C for 5 min. Then, 200 μl of α-thrombin solution was added to 100 μl of factor II–deficient human plasma in a cuvette. Clotting time was measured with a Behring Fibrintimer at 37°C.

Platelet Aggregation—Washed human platelets were prepared by centrifuging citrated whole blood and resuspending the platelets to a concentration of 1.3×10^8 per ml in Tyrode's buffer containing 0.35% human serum albumin at pH 7.3. Aggregation was initiated by adding 100 μl aliquots of α-thrombin at various concentrations in 50 mM Tris-HCl, 0.1% PEG 8000, 0.01% human serum albumin, and 100 mM NaCl, pH 7.5, to aliquots of platelet suspension (300 μl). The changes in light transmission were measured in an aggregometer at 37°C. We defined aggregation time as the time until complete aggregation of the platelets, as measured by the transmission of light through the suspension.

RESULTS

Expression and Extraction of Recombinant Prethrombin-2—The prethrombin-2 gene from human liver mRNA was cloned into pUT1, an expression vector with the *trc* promoter derived from pKK233-2 and *ori* derived from pUC18 (Fig. 1). Prethrombin-2 expression was induced by IPTG. Recombinant prethrombin-2 produced in *E. coli* was obtained as inactive and insoluble inclusion bodies. From 1 liter of *E. coli* cell culture, 500 mg (wet weight) of purified inclusion bodies (containing about 100 mg of protein) was obtained. The inclusion body fraction purified as described in "EXPERIMENTAL PROCEDURES" was observed as a major single band of prethrombin-2 on reducing SDS-PAGE (Fig. 2, lane 2).

Refolding of Recombinant Prethrombin-2—The inclusion bodies could be solubilized by adding 30 mM cysteine and 6 M guanidine hydrochloride, pH 9.5, to a final concentration of 10–25 mg/ml (containing about 2–5 mg/ml of protein). To determine the optimum refolding conditions, the effects of pH, temperature, and additives in the refolding buffer were studied (Table I). The pH was optimized within the range of pH 7 to 11. For optimizing the reconstitution conditions, the following additives were considered: (i) various concentrations of electrolytes and organic solvents, including NaCl, $CaCl_2$, 2,2,2-trifluoroethanol, 2-propanol, methanol, *etc.*, to alter the dielectric constant of the refolding buffer; (ii) low concentrations of denaturants such as arginine, guanidine hydrochloride, urea, *etc.*, to weaken inappropriate inter-molecular interactions; (iii) solvent components to stabilize folding intermediates, such as ammonium sulfate, sucrose, glycerol, and PEG 3350 and 8000; (iv) the addition of various detergents such as Tween-20 and -80, Brij-35 and -58, and Triton X-100 and -405 to prevent the adsorption of folding intermediates to the walls of the vessels used for refolding; and (v) disulfide bond formation by oxido-shuffling systems, such as reduced glutathione (GSH) and glutathione disulfide (GSSG), cysteine and cystine. The results were as follows: The addition of electrolytes such as NaCl and $CaCl_2$ was effective, especially high concentrations of NaCl (over 0.5 M), but the addition of organic solvents was not effective. Arginine concentrations above 0.5 M were more effective than guanidine hydrochloride, while urea was not effective. Glycerol at a concentration of 5–10% was effective, but other chemicals were less effective. Finally, the addition of more than 0.2% Brij-58 was effec-

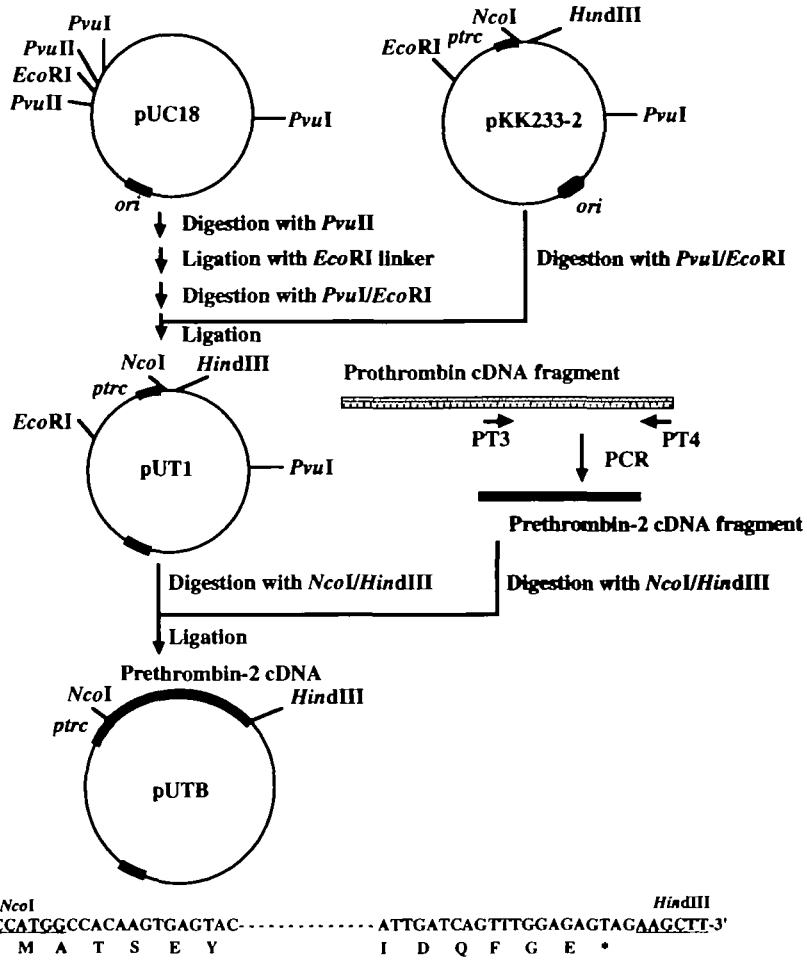

Fig 1 **Construction of an expression vector for prethrombin-2.** pUT1 was constructed from the DNA fragment including *ori* of pUC18 and the *trc* promoter of pKK233-2. The prothrombin fragment was amplified from cDNA of human liver mRNA. The expression vector was made by subcloning the *Nco*I–*Hin*dIII fragment encoding prethrombin-2 into pUT1

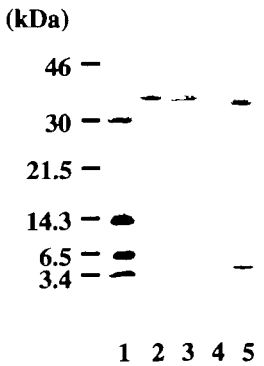

Fig. 2 **SDS-PAGE analysis of recombinant and plasma-derived thrombins.** About 1 μg of each protein was subjected to SDS-PAGE in a 15–25% polyacrylamide gradient gel under reducing conditions The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight markers; lane 2, isolated inclusion body; lane 3, purified refolded recombinant prethrombin-2, lane 4, purified recombinant α-thrombin; lane 5, plasma-derived α-thrombin; lane 6, *N*-glycosidase F-treated plasma-derived α-thrombin Treatment with *N*-glycosidase F was performed as follows. Plasma-derived α-thrombin (10 μg) was incubated with 1 unit of *N*-glycosidase F overnight at 37°C in 50 mM phosphate buffer, pH 8 6, containing 50 mM benzamidine-HCl and 10 mM EDTA.

tive, but none of the other detergents examined was effective. Finally, the effect of the addition of 1 mM reduced glutathione (GSH) was equal to that of L-cysteine, and the subsequent addition of one-tenth in the oxide form (GSSG and L-cystine, respectively) was slightly effective. The above effects were additive rather than synergistic. Finally, the solubilized protein was most efficiently refolded by single-step dilution in refolding buffer [50 mM Tris-HCl containing 20 mM $CaCl_2$, 500 mM NaCl, 1 mM EDTA, 600 mM arginine, 1 mM cysteine, 0 1 mM cystine, 10% (v/v) glycerol, and 0.2% (w/v) Brij-58, pH 8.5] at room temperature. Furthermore, refolding at room temperature was about 2-fold more effective than that at 4°C (data not shown). The yield of refolded prethrombin-2 was about 4–7% of the starting amount of solubilized protein. On the other hand, DiBella *et al.* (*8*) reported that during the refolding step, sulfonation of bovine prethrombin-2 and the addition of a strong denaturant, such as urea or guanidine hydrochloride, are important for effective refolding and that, in this case, the refolding efficiency is 1–4%. The yield of refolded protein using our method was about 2-fold higher than the yield using the method reported by DiBella *et al.* (*8*).

Purification of Recombinant Prethrombin-2—The refolded protein was dialyzed against 20 mM citrate buffer, pH 5.5, at 4°C, then centrifuged and filtered to remove the generated precipitate. Subsequently, to concentrate the protein solution, the folding mixture was subjected to SP-Sepharose chromatography, and finally purified by hirudin-based COOH-terminal peptide affinity chromatography. The puri-

TABLE I **Summary of screening for refolding conditions of recombinant prethrombin-2 from inclusion bodies.**

Additives[a]	Conditions
NaCl	0.02 to 3.0 M
$CaCl_2$	0.02 to 0.2 M
2,2,2-Trifluoroethanol	0.01 to 25%
2-Propanol	0.02 to 2%
Methanol	0.02 to 2%
Acetone	0.02 to 2%
Dimethyl sulfoxide	0.02 to 2%
Formamide	0.02 to 2%
L-Arginine[b]	0.05 to 2.0 M
L-Lysine[b]	0.5 M
Glycine[b]	0.5 to 1.0 M
Guanidine hydrochloride[b]	0.25 to 5.0 M
Urea[b]	0.5 to 2.0 M
Ammonium sulfate	0.05 to 2.0 M
Sucrose	0.1 to 0.5 M
Glycerol	0.5 to 40%
Polyethyleneglycol 3350	0.1 to 8%
Polyethyleneglycol 8000	0.1 to 8%
Tween-20	0.05 to 5%
Tween-80	0.05 to 5%
Brij-35	0.05 to 5%
Brij-58	0.05 to 5%
Triton X-100	0.05 to 5%
Triton X-405	0.05 to 5%
Reduced glutathione (GSH)	0 to 10 mM
Glutathione disulfide (GSSG)	0 to 1.0 mM
L-Cysteine	0 to 10 mM
L-Cystine	0 to 1.0 mM

[a]With the exception of some modifications, refolding was initiated by diluting the solubilized protein in the base buffer (50 mM Tris-HCl, pH 8.5, containing 0.5 M arginine, 20 mM NaCl, 20 mM $CaCl_2$, 1 mM EDTA, 1 mM cysteine, and 10% glycerol) containing the additives, at room temperature, followed by incubation overnight at room temperature. [b]Base buffer was 50 mM Tris-HCl, pH 8.5, containing 20 mM NaCl, 20 mM $CaCl_2$, 1 mM EDTA, 1 mM cysteine, and 10% glycerol

fied protein migrated as a single band with an apparent molecular mass of 36,000 Da on reducing SDS-PAGE, which is close to the calculated molecular mass of 35,400 Da (Fig. 2, lane 3). The final yield of purified prethrombin-2, based on the absorbance at 280 nm [estimated using the extinction coefficient $\varepsilon_{1\%} = 18.3$ (*20*)], was 0.5–1% of the starting amount of solubilized protein and about 1 mg/liter cell culture.

Activation and Purification of Recombinant Protein—Ecarin, a prothrombin activator from snake venom (*21*), was used to activate prethrombin-2 to α-thrombin. The resulting activated protein (cleaved at the Arg320-Ile321 bond) was purified by hirudin-based COOH-terminal peptide affinity chromatography. Ecarin-activated α-thrombin can be separated from the inactivated form, prethrombin-2, by changing the elution conditions in hirudin peptide affinity chromatography as described in "EXPERIMENTAL PROCEDURES." On reducing SDS-PAGE, the recombinant α-thrombin thus obtained showed two bands originating from the A chain and B chain with apparent molecular masses of about 4,000 and 30,000 Da, respectively. In addition, no further proteolyzed forms, such as β-thrombin (generated by cleavage at Arg382 and Arg393), or γ-thrombin (generated by cleavage at Arg382, 393, 443, and Lys474) (*4*) were detected (Fig. 2, lane 4).

Comparison of the Physicochemical Properties of Recombinant and Plasma-Derived α-Thrombins—Reducing SDS-PAGE analysis showed no difference in the mobilities of the A chains between recombinant and plasma-derived α-thrombins, but there was a difference in the mobilities of the B chains (Fig. 2, lanes 4 and 5). To determine whether this difference is due to *N*-linked carbohydrate chains, plasma-derived α-thrombin was treated with *N*-glycosidase F, an enzyme that removes *N*-linked carbohydrate chains from protein substrates (*22*). Following this treatment, both recombinant and plasma-derived α-thrombin B chains gave rise to products of identical size corresponding closely in molecular weight to the predicted peptide molecular mass of 30,000 Da (Fig. 2, lanes 4 and 6).

Furthermore, we analyzed the NH_2-terminal amino acid sequences of the isolated A and B chains from recombinant and plasma-derived α-thrombin, respectively. The sequences of the first 5 amino acid residues on each of the A and B chains showed no difference between recombinant and plasma-derived α-thrombins. As expected, the B chain sequence, Ile-Val-Glu-Gly-Ser, corresponded to the NH_2-terminal amino acid residues 321–325 of thrombin, *i.e.* part of the factor Xa cleavage site. On the other hand, the NH_2-terminal amino acid sequence of both A chains, Thr-Phe-Gly-Ser-Gly, showed a deletion of the first 13 residues from Arg271-Thr272 in the factor Xa cleavage site, as reported by Downing *et al.* (*23*).

Moreover, the results of active site titration indicated that each α-thrombin had more than 90% activity. Thus, the active site concentrations of recombinant and plasma-derived α-thrombins were close to the total protein concentrations, suggesting that nearly all of each α-thrombin was in an active form.

Comparison of the Enzymatic Properties of Recombinant and Plasma-Derived α-Thrombins—We compared the enzymatic properties of recombinant α-thrombin with those of plasma-derived α-thrombin in several assays. First, the steady-state parameters for the hydrolysis of the chromogenic substrate S-2238 were compared for recombinant and plasma-derived α-thrombins, with advance confirmation that they had the same pH and temperature dependencies for the hydrolysis of S-2238 (data not shown). As shown in Table II, the k_{cat} (174 ± 6 s^{-1}) and K_m (8.5 ± 0.2 μM) determined for the hydrolysis of S-2238 by recombinant α-thrombin are in reasonable agreement with the k_{cat} (174 ± 15 s^{-1}) and K_m (7.1 ± 0.5 μM) determined for plasma-derived α-thrombin.

We also compared the kinetic parameters for the release of FPA from fibrinogen, the activation of protein C in the absence and presence of thrombomodulin, and the activation of TAFI in the presence of thrombomodulin. The FPA release data provided a good fit to the first-order rate equation. The values of k_{cat}/K_m were calculated as the rate constant divided by the α-thrombin concentration. As shown in Table II, the value of k_{cat}/K_m for FPA release from fibrinogen is in good agreement with that of FPA release by plasma-derived α-thrombin (k_{cat}/K_m by recombinant α-thrombin = 12.5 ± 0.6 μM^{-1} s^{-1} and by plasma-derived α-thrombin = 10.6 ± 0.8 μM^{-1} s^{-1}). The k_{cat} and K_m values for protein C activation with/without GAG-UTM [glycosaminoglycan-modified urinary thrombomodulin (*9*, *10*)] were also determined. In each case, the plots of the rate of protein C activation against protein C concentration provided a good fit to the Michaelis-Menten equation. In the absence

TABLE II **Comparison of the functional activities of recombinant and plasma-derived α-thrombins.** All data represent the means of three experiments ± standard deviation

Assay	Recombinant α-thrombin	Plasma-derived α-thrombin
Hydrolysis of S-2238		
k_{cat} (s^{-1})	174 ± 6	174 ± 6
K_m (μM)	8.5 ± 0 2	7 1 ± 0 5
k_{cat}/K_m (μM^{-1} s^{-1})	20.3 ± 0 4	24 5 ± 0 6
Release of FPA		
k_{cat}/K_m (μM^{-1} s^{-1})	12.5 ± 0 6	10 6 ± 0 8
Dissociation constant for GAG-UTM		
K_{dapp} (nM)	1 1 ± 0 2	1 4 ± 0.2
Activation of protein C without GAG-UTM		
k_{cat} (min^{-1})	10 3 ± 0.1	8.5 ± 0 2
K_m (μM)	11 3 ± 0.3	16 3 ± 0.3
k_{cat}/K_m (μM^{-1} min^{-1})	0.91 ± 0.02	0 52 ± 0.02
Activation of protein C with GAG-UTM		
k_{cat} (min^{-1})	111 ± 6	97 7 ± 2 8
K_m (μM)	6 7 ± 0 5	5 9 ± 0 4
k_{cat}/K_m (μM^{-1} min^{-1})	16 6 ± 0 2	16 7 ± 0 7
Activation of TAFI with GAG-UTM		
k_{cat}/K_m (μM^{-1} s^{-1})	2 4 ± 0 2	2 1 ± 0 1
Inhibition by AT III		
Second order rate constant (μM^{-1} min^{-1})	0 62 ± 0 01	0.65 ± 0 01

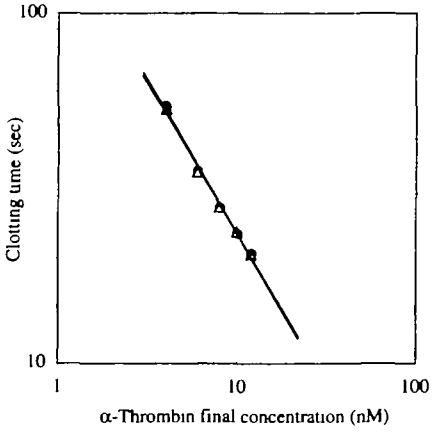

Fig. 3 **Comparison of recombinant and plasma-derived α-thrombins for clotting activity using factor II–deficient human plasma.** Aliquots of 200 μl of various concentrations of recombinant (△) or plasma-derived (○) α-thrombin ranging from 6 nM to 18 nM in the presence of 5 mM $CaCl_2$ were added to 100 μl of factor II–deficient human plasma in cuvettes. Final concentrations of α-thrombins ranged from 4 to 12 nM Clotting time was measured with a Behring Fibrintimer at 37°C

of GAG-UTM, as shown in Table II, the value of k_{cat}/K_m for protein C activation by recombinant α-thrombin was slightly higher than that of plasma-derived α-thrombin (k_{cat}/K_m by recombinant α-thrombin = 0.91 ± 0.02 μM^{-1} min^{-1} and by plasma-derived α-thrombin = 0.52 ± 0.02 μM^{-1} min^{-1}). This is mainly due to a reduction in the K_m for protein C in recombinant α-thrombin (K_m in recombinant α-thrombin = 11.3 ± 0.3 μM and in plasma-derived α-thrombin = 16.3 ± 0.3 μM). In the presence of GAG-UTM, the apparent dissociation constants (K_{dapp}) for GAG-UTM of both α-thrombins were determined in advance. As shown in Table II, the K_{dapp} of recombinant α-thrombin is similar to that of plasma-derived α-thrombin (the former is

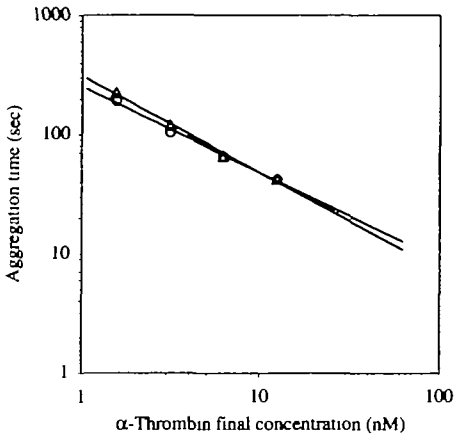

Fig 4 **Comparison of recombinant and plasma-derived α-thrombins for platelet aggregation using washed human platelets.** Aliquots of 300 μl of washed human platelets resuspended to a concentration of 1 3 × 10^8 platelets per ml were mixed with 100 μl of various concentrations of recombinant (△) or plasma-derived (○) α-thrombin The changes in light transmission were measured in an aggregometer at 37°C We defined aggregation time as the time until complete aggregation of the platelets, as measured by the transmission of light through the suspension.

1.1 ± 0 2 nM, and the latter 1.4 ± 0.2 nM). For protein C activation, in the presence of GAG-UTM, the values of k_{cat} (111 ± 6 min^{-1}) and K_m (6.7 ± 0.5 μM) by recombinant α-thrombin are in good agreement with the k_{cat} (97.7 ± 2.8 min^{-1}) and K_m (5.9 ± 0.4 μM) determined for plasma-derived α-thrombin (Table II).

Furthermore, the values of k_{cat}/K_m for the activation of TAFI in the presence of GAG-UTM were determined. As shown in Table II, the value of k_{cat}/K_m for TAFI activation by recombinant α-thrombin (2.4 ± 0.2 μM^{-1} s^{-1}) is close to that of plasma-derived α-thrombin (2.1 ± 0.1 μM^{-1} s^{-1}).

To assess the reactivity of recombinant α-thrombin to a natural protein inhibitor, the rate of inactivation by AT III was compared (Table II). The second-order rate constant for the AT III–mediated inhibition of recombinant α-thrombin (0.62 ± 0.01 μM^{-1} min^{-1}) is similar to that of plasma-derived α-thrombin (0 65 ± 0.01 μM^{-1} min^{-1}).

Finally, we compared the procoagulant activity and the ability to form platelet aggregates of recombinant α-thrombin with those of plasma-derived α-thrombin. The clotting activity of recombinant α-thrombin, using factor II–deficient human plasma, was found to be in good agreement with that of plasma-derived α-thrombin (Fig. 3). Furthermore, using washed human platelets, the proaggregatory activity of recombinant α-thrombin with that of plasma-derived α-thrombin was tested under the conditions described in "EXPERIMENTAL PROCEDURES." As shown in Fig. 4, the plots of aggregation time *versus* α-thrombin concentration for both recombinant and plasma-derived α-thrombin show good fits with the linear regression line, and the results for both are in reasonable agreement with each other.

DISCUSSION

α-Thrombin has a variety of properties and interactions, acting as both a procoagulant and anticoagulant (*4*). For

instance, fibrinogen undergoes limited proteolysis by thrombin (cleavage of the Arg-Gly bond in each of the Aα and Bβ chains, releasing fibrinopeptides A and B, respectively) in one of the final steps of blood coagulation (*24*). The resulting fibrin monomers are polymerized into a fibrin clot, which is then cross-linked and stabilized by factor XIIIa, a transglutaminase activated by thrombin (*25*, *26*). Thrombin also activates blood coagulation factors V (*27*), VIII (*28*), and XI (*29*) in a feedback pathway. By forming a complex with the endothelial cell-surface protein thrombomodulin, thrombin can also activate thrombin-activatable fibrinolysis inhibitor (TAFI) (*30*) and anticoagulant protein C (*31*). Thrombomodulin acts as a regulatory factor that alters the specificity of thrombin from its preferred substrate fibrinogen in the procoagulation process to protein C in the anticoagulation process (*32*). By activating thrombin receptors on the surface of platelets, thrombin can stimulate platelet aggregation (*33*). These activities of thrombin can be controlled by serine protease inhibitors, such as AT III and heparin cofactor II (HC II) (*34*, *35*). Thrombin is thus a multifunctional protease and is used as a pharmaceutical reagent, *e.g.* as an aid in hemostasis. Thrombin has to be carefully prepared from plasma, because plasma-derived reagents, according to circumstances, have possible disadvantage of being contaminated by other clotting agents or various pathogens.

We have developed a new method for the expression and refolding of human prethrombin-2 using *E. coli*, which is suitable for the large-scale production of recombinant α-thrombin. The expressed prethrombin-2 was produced as inclusion bodies. It has been reported that the solubilization and refolding of prethrombin-2 from inclusion bodies is difficult (*6–8*). Various refolding conditions of inclusion bodies, as shown in Table I, were considered: (i) various concentrations of electrolytes and organic solvents; (ii) low concentrations of denaturant; (iii) solvent components to stabilize folding intermediates; (iv) addition of various detergents; and (v) disulfide bond formation by oxido-shuffling. The present method made it possible to refold inclusion bodies in a simple one-step dilution process at pH 8.5 in buffer consisting of 50 mM Tris-HCl, 20 mM $CaCl_2$, 500 mM NaCl, 1 mM EDTA, 600 mM arginine, 1 mM cysteine, 0.1 mM cystine, 10% (v/v) glycerol, and 0.2% (w/v) Brij-58 at room temperature. The yield of refolded prethrombin-2 (the enzymatically intact precursor of thrombin) was about 4–7% of the starting amount of solubilized protein. In addition, the final yield of highly purified refolded protein was 0.5–1%, and about 1 mg of recombinant protein could be isolated from 1 liter of *E. coli* cell culture. The refolded recombinant prethrombin-2 could be purified by affinity chromatography on a hirudin-based C terminal peptide immobilized column. This suggests that the fibrinogen recognition exosite in the refolded molecules is intact, since hirudin accommodates itself to the restricted active site with concomitant binding of acidic residues to the basic fibrinogen recognition exosite (*36*). This finding was also supported by the observation that the rates of FPA release from fibrinogen by plasma-derived and recombinant α-thrombins were indistinguishable from each other (Table II).

The refolded recombinant prethrombin-2 was fully activated by ecarin under the conditions described above, and the generated recombinant α-thrombin showed similar physicochemical properties to plasma-derived α-thrombin. On reducing SDS-PAGE (Fig. 2, lanes 4–6) and NH_2-terminal sequence analyses, no differences were detected between recombinant and plasma-derived α-thrombins, except for the absence of the *N*-linked carbohydrate chains in the *E. coli*-derived recombinant protein. However, the *N*-linked glycosylation site of plasma-derived α-thrombin is Asn373, which is located in the 60-loop [numbered according to the chymotrypsinogen numbering system (*37*)]. This loop caps the active site (*4*) and plays a major role in the restricted specificity of thrombin (*38*). Therefore, it is important to compare the enzymatic activities of recombinant and plasma-derived α-thrombins for various protein substrates. The enzymatic properties of our recombinant α-thrombin showed reasonable agreement with those of plasma-derived α-thrombin (Table II). Furthermore, the results of experiments on clotting activity and platelet aggregation also showed good agreement between recombinant and plasma-derived α-thrombin (Figs. 3 and 4). These results suggest that the *N*-linked carbohydrate chain in the 60-loop within the B chain of human plasma-derived wild-type α-thrombin may not affect its physiological functions. This finding is consistent with the case of bovine α-thrombin (*8*).

In conclusion, the refolding method described in this report is a simple and efficient way to obtain pathogen-free recombinant human α-thrombin, which could be used not only as a biochemical reagent, but also as a pharmaceutical reagent.

We would like to thank Professor Izumi Kumagai, and Dr Kouhei Tsumoto (Tohoku University) for their helpful discussions on the folding of proteins We would also like to thank Professor Sadaaki Iwanaga (Fujita Health University) for his many helpful discussions and critical reading of the manuscript, as well as Drs. Kazuhiro Onoki and Toshiyuki Edano for supplying the GAG-UTM We would also like to thank Dr Tomohiro Nakagaki for supplying the plasma-derived protein C and activated protein C, Tetsuro Tanabe and Keishin Sugawara for supplying large scale inclusion bodies, and Kazuhiko Tomokiyo and Kaori Teshima for their technical assistance

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