Construction, properties and specific fluorescent labeling of a bovine prothrombin mutant engineered with a free C-terminal cysteine

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To define the role of phosphatidylserine-induced conformational changes in prothrombin activation during blood coagulation, a recombinant bovine prothrombin was constructed, characterized and shown to have a globally native-like conformation. We introduced a cysteine to replace the penultimate residue (Gly581) of a previously constructed active site mutant, and expressed the double mutant in Chinese hamster ovary cells at the level of 0.6 μg/ml of cell culture medium. Specific labeling with fluorescein maleimide was accomplished by limited reduction with dithiothreitol to free the engineered cysteine while maintaining the native-like functional properties of the molecule. The average stoichiometry of labeling was 0.84 probe/protein. The location of the probe at the C-terminus was confirmed by proteolysis by native thrombin, by Taipan venom, and by carboxypeptidase Y. Both the double mutant and labeled prothrombin could be activated by snake venoms and the prothrombinase but, as expected, the double mutant meizothrombin did not autolyze as does native meizothrombin. Thus, for the first time, a native-like but specifically labeled prothrombin has been constructed. This molecule will be an essential tool for elucidating the structural role of membranes during prothrombin activation. In addition, the methods described might be usefully applied to labeling of an odd, engineered cysteine in other disulfide bond-containing proteins.

Keywords: fluorescent label/limited reduction/recombinant prothrombin

Introduction

Thrombin is the central regulatory enzyme in the blood coagulation process. This key serine protease and regulatory molecule circulates in blood as an inactive zymogen, prothrombin (II). Prothrombin activation to thrombin is accomplished physiologically by the prothrombinase complex, composed of factor Xa, factor Va, Ca2+ and a negatively charged phospholipid membrane surface, probably supplied, in vivo, by platelet membranes (Mann et al., 1988). In this complex, factor Xa serves as a serine protease to cleave two peptide bonds of prothrombin. As shown in Figure 1, the cleavage at Arg323–Ile (3) exposes the active site of the protease domain of prothrombin and gives rise to meizothrombin, the active intermediate of prothrombin activation. The other cleavage at Arg274–Thr (2) releases the protease domain (thrombin) from fragment 1.2, the membrane-bound N-terminal part of prothrombin (see Figure 1). We have suggested that a difference in the phosphatidylserine (PS)-induced conformations of mem-

brane-bound prothrombin and meizothrombin is crucial to the role of platelet membranes in blood coagulation (Pei et al., 1992; Lentz et al., 1994; Wu and Lentz, 1994). To detect this conformational difference, a specifically labeled prothrombin is needed.

The use of amino acid and peptidyl chromomethyl ketone as irreversible inhibitors of serine protease was pioneered by Shaw and co-workers (Kettner and Shaw, 1981). Such active-site inhibitors have been used to label specifically serine proteases or their active intermediates, such as thrombin and meizothrombin, by attaching fluorophores to the N-terminus of the peptides (Husten et al., 1987; Williams et al., 1989; Armstrong et al., 1990). Although a simple and powerful method for obtaining labeled protease, the shortcoming of this method is that the label can only be located at the active site of the protease, which is not present in the zymogen, such as prothrombin.

In the study reported here, specific labeling of a recombinant bovine prothrombin was achieved by introducing a cysteine to replace the penultimate residue (Gly581) at the C-terminal end of a previously constructed active site mutant prothrombin [P(S528A); Pei et al., 1991]. There are a total of 12 disulfide bonds in bovine prothrombin, four of these being in the catalytic domain. We chose the C-terminal site based on a proposed model of prothrombin (Armi et al., 1994). This model indicated that the C-terminus is accessible and that the catalytic domain disulfide cysteine pairs are buried inside the protein, far away from the C-terminus. By this choice, we hoped to minimize disulfide isomerization that might be associated with an engineered odd cysteine residue. We describe in this paper the design, cloning, expression, isolation, labeling and enzymatic characterization of this double mutant prothrombin [P(S528A,G581C)]. We show that P(S528A,G581C) was expressed in practical levels, could be isolated in pure form, could be labeled at the engineered cysteine with fluorescent probes and displayed reasonably native-like properties, nearly identical with those of the single mutant from which it was derived. We conclude that this engineered prothrombin will be a very useful molecule in elucidating structural features of the enzymatic process.

Materials and methods

Materials

Bovine brain phosphatidylserine (PS) and 1-palmitoyl-2-oleoyl-3-sn-phosphatidylcholine (POPC) were purchased from Avanti Biochemicals (Birmingham, AL, USA). Dansyl-arginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was synthesized and purified according to Mann et al. (1981). D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride (S2238) was from Chromogenix (Molndal, Sweden). Ecarin from Echis carinatus snake venom and Taipan snake venom from Oxyuranus scutellatus were purchased from Sigma Chemical (St Louis, MO, USA). Cell growth medium, Dulbecco's modified Eagle's medium/F12 (DMEM/F12), was
Fig. 1. Fragments of bovine prothrombin generated by factor Xa and thrombin. Factor Xa is a serine protease in the prothrombinase complex that cleaves two peptide bonds of prothrombin. The cleavage at the Arg323—lie peptide bond (site 3) exposes the active site of the protease domain of prothrombin and gives rise to an active intermediate of prothrombin activation, meizothrombin. The other cleavage at the Arg274-Thr peptide bond (site 2) releases the protease domain (thrombin) from fragment 1.2, the membrane-bound N-terminal part of prothrombin. Bovine prothrombin can also be digested by native bovine thrombin or bovine meizothrombin at the Arg155—Ser peptide bond to generate fragment 1 and prethrombin 1.
of serum-free medium. Approximately 150 ml of conditioned medium were harvested every 5–7 days, with 150 ml of fresh serum-free medium added, for a total harvesting period of 5–7 weeks. We routinely maintained 20 roller bottle cultures to produce the double mutant prothrombin. Inhibitor cocktail (15 ml of 1 M benzamidine, 85 ml of 48.8 mM phenylmethylsulfonyl fluoride in 2-propanol) was added (1–100 ml) to the harvested medium. The harvested medium was precipitated with 100 mM barium chloride, 15 mM sodium citrate and 2 mM benzamidine at 4°C, and the precipitate was frozen at –70°C until several such precipitates could be pooled for isolation of prothrombin. Because precipitation by barium citrate is specific for plasma proteins containing N-terminal domains rich in post-translationally carboxylated glutamate, this single step separates properly carboxylated prothrombin from most secreted cell proteins (Pei et al., 1991).

Isolation of P(S528A,G581C)

The pooled precipitate was washed four times with 0.1 M barium chloride and 1 mM benzamidine, resuspended in 40% saturated ammonium sulfate and centrifuged at 4000 r.p.m. in a Sorvall RC-3B refrigerated centrifuge (Du Pont, Wilmington, DE, USA) for 20 min. The supernatant was dialyzed overnight against 20 mM Tris–HCl, 1 mM benzamidine (pH 7.4) at 4°C, and loaded onto a DEAE Sephacel column that had been equilibrated with 20 mM Tris–HCl, 100 mM NaCl, 2 mM benzamidine (pH 7.4) buffer. The protein was eluted with 20 mM Tris–HCl, 500 mM NaCl, 2 mM benzamidine (pH 7.4) buffer. Protein absorbance was monitored at 280 nm and protein concentration was determined using an extinction coefficient of 1.44 OD/(mg/ml) for prothrombin (Mann, 1976). The yield of the double mutant prothrombin was approximately 0.6 μg/ml cell culture medium. The protein-containing fractions were then pooled and applied to an HPLC Mono Q ion-exchange column (HR 5/5, Pharmacia LKB Biotechnology, Uppsala, Sweden). After loading the sample, the column was washed with 20 mM Tris–HCl (pH 7.4) buffer and the protein eluted with a linear gradient from 0 to 0.7 M NaCl in the same buffer at a flow rate of 1.5 ml/min. The identities of proteins in different fractions were determined by SDS–PAGE (10% acrylamide, Laemmli system), using low range SDS–PAGE molecular weight markers (Bio-Rad) and purified native prothrombin as the standards. The fractions containing pure prothrombin were combined and stored at –70°C in 20 mM Tris–HCl, 350 mM NaCl (pH 7.4) buffer.

Prothrombin activation by prothrombinase and enzymatic activity of the activation products

The P(S528A,G581C), P(S528A) and native prothrombin (all at 1.6 μM) were activated in a reaction mixture containing 0.5 mM factor Xa, 5 nM factor Va, 10 μM PS/POPC (25/75) SUV and 5 mM CaCl2 in 20 mM Tris–HCl, 150 mM NaCl (pH 7.4) at room temperature. At intervals of 0, 5 and 10 min, aliquots were withdrawn and subjected to SDS–PAGE (10% acrylamide, Laemmli system) under non-reducing and reducing (3% 2-mercaptoethanol) conditions. Gels were stained with Coomassie Blue R-250 (Sigma) and the protein bands were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA, USA) to estimate the initial rate of prothrombin activation.

Activation proceeded to completion at 37°C in 45 min. The product, identified by SDS–PAGE, was thrombin in every case. An aliquot from each incubation was taken to measure the activity toward synthetic chromogenic substrate S2238 (Chromogenix) and compared with native thrombin. The

Fig. 2. Maps of p487 and p718. p718 is the shuttle expression vector to express Ser528Ala and Gly581Cys double mutant bovine prothrombin [P(S528A,G581C)], which was constructed from p487, the vector for P(S528A), by site-directed mutagenesis.
Fig. 3. SDS–PAGE analysis of recombinant prothrombin activation by the full prothrombinase complex. Recombinant and native prothrombin (1.6 μM) were activated to thrombin with prothrombinase at room temperature. At intervals of 0, 5 and 10 min, aliquots were withdrawn and subjected to 10% SDS–PAGE under non-reducing and reducing (3% of 2-mercaptoethanol) conditions. Lanes 1, 2 and 3 are native bovine prothrombin, lanes 4, 5 and 6 are single mutant recombinant prothrombin [P(S528A)] and lanes 7, 8 and 9 are double mutant recombinant prothrombin [P(S528A,G580C)]. The bands indicated under non-reducing conditions are prothrombin or meizothrombin, prethrombin 1, thrombin, fragment 1-2 and fragment 1. The bands indicated under reducing conditions are prothrombin, prethrombin 1, fragment 1-2 with or without thrombin A chain, thrombin B chain, fragment 1 and fragment 2.

Fig. 4. SDS–PAGE analysis of recombinant prothrombin activation by ecarin. Ecarin (final concentration 36 μg/ml) was added to 90 μg/ml of native or recombinant prothrombin to start the activation. At 0, 30 and 90 min, aliquots were withdrawn and subjected to 10% SDS–PAGE under both non-reducing and reducing conditions (3% of 2-mercaptoethanol). Lanes are indicated as in Figure 3. The bands indicated under non-reducing conditions are prothrombin or meizothrombin, meizothrombin des fragment 1 and fragment 1. The bands indicated under reducing conditions are prothrombin, prethrombin 1, fragment 1-2 with thrombin A chain, thrombin B chain and fragment 1.

measurements were performed simultaneously on a 96-well plate with a microplate reader (Model 340 ATTC, SLT, Hillsborough, NC, USA) with absorbance read every 5 s at 405 nm. For the synthetic chromogenic substrate assay, 5 μl aliquots (containing 0.45 μg of prothrombin), 40 μl of 1 mM S2238 and 105 μl of 38 mM Tris–HCl, 150 mM NaCl, 0.6% PEG-8000 (pH 8.3) buffer were used.

Prothrombin activation by ecarin and Taipan venom
Recombinant prothrombin was activated with ecarin from Echis carinatus venom, which cleaves the Arg323–Ile bond of bovine prothrombin to yield meizothrombin (Rosing and Tans, 1988). Ecarin (36 μg/ml final concentration) was added to a final concentration of 90 μg/ml of native or recombinant prothrombin in 20 mM Tris–HCl, 150 mM NaCl, 6 mM CaCl₂ (pH 7.4) buffer at 37°C to start the activation. At intervals of 0, 30 and 90 min, aliquots were withdrawn and subjected to SDS–PAGE (10% acrylamide) under both non-reducing and reducing conditions. Gels were stained with Coomassie Blue R-250.

A DAPA fluorescence assay (Mann et al., 1981; Nesheim and Mann, 1983; Krishnaswamy et al., 1986) was used to compare the activation of mutant and native prothrombin by ecarin and Taipan venom. Using an SLM 48000 MHF fluorimeter (SLM Instruments, Urbana, IL, USA) with excitation wavelength at 280 nm and a 515 nm cut-off emission filter,
we measured emission of DAPA at 37°C for the reactions of 125 nM mutant or native prothrombin, 10 µM PS–POPC (25:75) SUV, 5 mM CaCl₂, and 500 nM DAPA in 20 mM Tris–HCl, 150 mM NaCl (pH 7.4) buffer.

**DAPA binding to mutant or native thrombin**

Mutant or native prothrombin (1.6 µM) was incubated in the dark with 50 µg/ml Taipan venom, 10 µM PS–POPC (25:75) SUV and 5 mM CaCl₂ in 20 mM Tris–HCl, 150 mM NaCl (pH 7.4) buffer at room temperature for 2 h. Prothrombin was completely activated to thrombin as judged by SDS–PAGE. The activation mixtures were then used to titrate 67 nM DAPA in 20 mM Tris–HCl, 150 mM NaCl (pH 7.4) at room temperature, with DAPA fluorescence recorded as described above. The data were analyzed by the following equation:

\[
\frac{1}{f} = \frac{[P]_0}{bK_d} - \frac{n[L]_0}{K_d}
\]

where \([P]_0\) and \([L]_0\) are the nominal concentrations of protein (thrombin) and ligand (DAPA), respectively, \(f\) and \(b\) are the fraction of total ligand concentration free and bound, respectively, \(n\) is the number of independent, non-interacting ligand binding sites per protein molecule and \(K_d\) is the site dissociation constant. The fraction of thrombin-bound ligand \(b\) was represented by the ratio of fluorescence intensity at a given addition of thrombin to the intensity at saturation. The fraction of free ligand was calculated as \(f = 1 - b\).

**Limited DTT reduction, fluorescent labeling and characterization of labeled P(S528A,G581C)**

Following purification through a DEAE-Sephaloc column (Sigma), the concentration of P(S528A,G581C) was estimated by absorbance measurements. Dithiothreitol (DTT) was added to the sample at [DTT]:[prothrombin] = 1.4:1 (mol/mol), and this mixture was incubated in the dark at room temperature for 1 h. DTT and benzamidine were removed by HPLC on a Perkin-Elmer Isopure LC system using a Mono Q HR 5/5 ion-exchange column (Pharmacia LKB). To decrease the buffer NaCl concentration to <150 mM, the protein sample was diluted fourfold with 20 mM Tris–HCl (pH 7.4), before loading it on the HPLC column. The elution profile was similar to that of non-reducing prothrombin, which eluted at 350 mM NaCl. The protein peaks from the Mono Q column were pooled and incubated in the dark at room temperature for 1 h. The prothrombin concentration was estimated by measuring the absorbance at 280 nm. A thio-specific fluorophore, fluorescein maleimide (FM), was dissolved in dimethyl sulfoxide at a concentration of 56 mM and added to the partially reduced prothrombin sample at a mole ratio of probe to protein of ~7:1. The final concentration of buffer was 350 mM NaCl, 20 mM Tris–HCl (pH 7.4) with the prothrombin concentration around 7 µM. The mixture was left in the dark at room temperature for 2 h.

The excess probe and high salt were removed by a desalting column (Excellulose GF-5, Pierce), and prothrombin was eluted with 100 mM NaCl, 20 mM Tris–HCl (pH 7.4). Incorporation of the fluorescent label and the purity of labeled prothrombin were determined by SDS–PAGE (10%) with fluorescence being detected by UV on FOTO/PREP II (FOTO-DYNE, New Berlin, WI, USA) and protein being determined by Coomassie Bright Blue staining.

To determine the probe–protein stoichiometry, the prothrombin concentration was determined by bicinchonic acid assay (BCA assay, Pierce). Since the BCA assay is quantitated by absorbance at 560 nm, this circumvented spectral interference from FM, whose concentration was detected by absorbance at 490 nm. Calibration graphs obtained with bovine serum albumin were used to quantitate both measurements.

A tricine–Tris–HCl SDS gel (16% acrylamide; Schagger and Jagow, 1987) was applied to identify the fluorescent labeled fragments in the prothrombin molecule.

**Results and discussion**

**Comparison of the functional properties of native, single and double mutant prothrombins**

The P(S528A,G581C), P(S528A) and native bovine prothrombin were all activated in a similar fashion by the fully assembled prothrombinase, as shown in the SDS–PAGE gel shown in Figure 3. The activation of both the single and double mutant prothrombin was slower than that of the native molecule under the same conditions. The initial rates of activation of the recombinant prothrombin, as estimated from scanning densitometry, were 35% for P(S528A) and 55% for P(S528A,G581C) relative to that for native prothrombin. There was no prothrombin 1 generated from the single or the double mutant prothrombin. Like all the serine protease, the active site of thrombin is composed of three critical residues: a His (366 in the bovine numbering system), an Asp (420) and a Ser (528). Therefore, the mutation of Ser528 to Ala leads to loss of thrombin activity towards both peptide and synthetic chromogenic (e.g. S2238) substrates (Pei et al., 1991). In order to confirm this, mutant prothrombin was activated to thrombin
at 37°C, and the activity of the mutant thrombin toward S2238 was compared with that of native thrombin. As expected, the thrombin from both the P(S528A,G581C) and P(S528A) displayed no amidolytic activity toward S2238 (data not shown).

The recombinant bovine prothrombin was also activated with purified ecarin from *Ecarinatus*, which cleaves the Arg323–Ile peptide bond of bovine prothrombin to expose the active site and form meizothrombin (Rosing and Tans, 1988). As judged by SDS-PAGE under reducing conditions, activation of both P(S528A,G581C) and P(S528A) was completed by 90 min, as was the activation of native prothrombin (Figure 4). However, in contrast to native prothrombin, the activation product of the mutant prothrombin was meizothrombin instead of the meizothrombin des fragment 1 produced from native prothrombin activation, confirming that the double mutant meizothrombin was not subjected to autolysis, as has already been shown for the single mutant molecule (Pei et al., 1991). The existence of a stable meizothrombin to which we can attach a fluorescent probe will provide opportunities to compare its structure and other characteristics with those of membrane-bound prothrombin.

DAPA is a specific, fluorescent inhibitor of thrombin. The fluorescence intensity of DAPA is greatly enhanced when bound to the active site of thrombin or its partially activated form, meizothrombin (Mann et al., 1981; Nesheim and Mann, 1983; Krishnaswamy et al., 1986). This allows the progress of prothrombin activation to be recorded continuously. Using the DAPA fluorescence assay, the activation time courses of mutant and native prothrombin by ecarin were compared (data not shown). In the presence of excess DAPA, activation of all three prothrombins is expected to lead, at least initially, to the meizothrombin intermediate. The time courses of activation were complex in shape, so we only determined the times required for maximal activation. They were 500, 600 and 800 s for native, single mutant and double mutant prothrombin, respectively. The maximum fluorescence intensities were 70 and 50% for single or double mutant prothrombin, respectively, compared with that of the native protein.

Unlike ecarin, crude Taipan venom activates prothrombin completely to thrombin. When monitored with the DAPA fluorescent assay (data not shown), the fluorescence intensity of DAPA in the case of P(S528A,G581C) activated by Taipan venom reached a plateau at 350 s, whereas native prothrombin and P(S528A) activation mixtures reached plateaus within 220 and 180 s, respectively. Compared with native prothrombin, the maximum fluorescence intensities were 70 and 45%, respectively.

The lower maximum fluorescence intensities of DAPA when bound to mutant thrombin or meizothrombin could be due either to reduced binding of DAPA to the mutated active site or to a reduced quantum yield of the DAPA–mutant thrombin or meizothrombin complex as compared with the DAPA–native thrombin or meizothrombin des fragment 1 complex. Pei *et al.* (1991) showed that single mutant prothrombin bound DAPA with a threefold higher $K_d$ than the native molecule and produced a complex with 74% of the fluorescence intensity of the native complex. In order to define the DAPA binding properties of the double mutant prothrombin, both native and double mutant prothrombin were completely activated to thrombin by Taipan venom as judged by SDS–PAGE. The activation mixtures were used to titrate 67 nM DAPA under the same conditions. Figure 5 shows that the fluorescence intensity of the DAPA–double mutant thrombin complex was 70% of that of the native complex but that $K_d$ was three times larger than that observed for the native molecule. Analysis by equation (1) gave the stoichiometry as $n = 0.97$ mol of DAPA per mole of native thrombin, $K_d = 3.9 \times 10^{-6}$ M, and $n = 0.91$ mol of DAPA per mole of native thrombin; $K_d = 1.3 \times 10^{-6}$ M. These values were nearly identical with binding parameters obtained by re-analysis of the single mutant data: $n = 0.82$ mol of DAPA per mole of single mutant thrombin.
no substantial conformational alteration occurred in was somewhat different. These observations suggest that DAPA bound to the activation products of the two mutants similar DAPA binding affinity, although the fluorescence of and P(S528A) are similar in that both have comparable or thrombin.

of DAPA with the exposed active site in either meizothrombin activation by prothrombinase components, or on the interaction at the C-terminus altered the interaction with and activation components. Our current results indicate that a modification might have altered interactions with one of the prothrombinase tional activity. It was also speculated that the active site mutant prothrombin were fully carboxylated and that the wild-type and single mutant recombinant recombinant and native molecules had nearly identical func-

et al., (1991) is in error, so data from that paper were re-analyzed according to equation (1) in this paper.

In both the enzymatic activation and DAPA binding measurements, the single and double mutants were similar to each other but somewhat different from the native molecule. The effect of the S528A on the development and properties of the active site were discussed in a previous paper (Pei et al., 1991). There, it was shown that the active site mutant might have altered interactions with one of the prothrombinase components, that the wild-type and single mutant recombinant prothrombin were fully carboxylated and that the wild-type recombinant and native molecules had nearly identical functional activity. It was also speculated that the active site mutant might have altered interactions with one of the prothrombinase components. Our current results indicate that a modification at the C-terminus altered the interaction with and activation by snake venoms but had little effect on interaction with and activation by prothrombinase components, or on the interaction of DAPA with the exposed active site in either meizothrombin or thrombin.

To summarize our property comparisons, P(S528A,G581C) and P(S528A) are similar in that both have comparable activation rates by the purified prothrombinase and both have similar DAPA binding affinity, although the fluorescence of DAPA bound to the activation products of the two mutants was somewhat different. These observations suggest that no substantial conformational alteration occurred in P(S528A,G581C) during biosynthesis. This is consistent with the proposal that disulfide bonds are formed between the appropriate cysteines in the most stable conformation of the protein (Speziale and Detwiler, 1990), leaving the odd, mutated cysteine at the penultimate residue unpaired. We conclude that little if any disulfide isomerization had been caused by the engineered odd cysteine in P(S528A,G581C), otherwise a significant change in the protein functional properties would be expected.

Limited reduction of the double mutant prothrombin by DTT

The engineered cysteine codon substitution at the penultimate position of P(S528A,G581C) has been confirmed by DNA sequencing. To our surprise, the double mutant prothrombin with the C-terminal cysteine was not labeled when incubated with an excess of thio-specific fluorescent reagents such as rhodamine-X iodoacetomide and fluorescein maleimide (FM).

Since bovine serum albumin, which has a free cysteine in the middle of the molecule, was successfully labeled under the same conditions, we concluded that the engineered cysteine residue in P(S528A,G581C) was not accessible. On the basis of the predicted structure of prothrombin (Arni et al., 1994), we expected the C-terminus of prothrombin to be on the surface of the protein molecule. This might lead to dimer formation between two P(S528A,G581C) molecules, but no high molecular weight protein band appeared in the P(S528A,G581C) lane of non-reducing SDS–PAGE gels, excluding this possibility. If our speculation about the surface location of the C-terminal cysteine was correct, and if the engineered cysteine was free, some dimer formation might have been expected. To test for the possibility that this odd C-terminal cysteine might be buried, we denatured the protein by heat and guanidine hydrochloride. These experiments showed that even in the denatured state the double mutant prothrombin was still not labeled with either FM or rhodamine-X iodoacetomide. Since the double mutant prothrombin retains essentially the functional properties of the single mutant, and since the double mutant was not labeled by any reagent directed towards free SH groups, even in the denatured state, we presumed that the sulfhydryl group in the penultimate residue would be blocked chemically via a soluble compound free in the cell (perhaps a free cysteine or reduced glutathione), rather than via disulfide isomerization.

To test for this possibility, we attempted to release the odd sulfhydryl group without damaging any of the 12 disulfide-linked cysteine pairs in the protein by adding various concentrations of DTT to samples containing native, single mutant or double mutant prothrombin. As shown in Figure 6, the fluorescence intensity, which indicated the extent of labeling, with 0.1 mM DTT was much less than that with 0.2 mM DTT. This suggested that limited reduction is possible. We then tested whether 0.1 mM DTT disrupted prothrombin structure owing to improper refolding or disulfide bond isomerization. Since uncatalyzed disulfide isomerization is normally a very slow reaction (Speziale and Detwiler, 1990), we expected that disulfide isomerization would be minimal under our experimental conditions. This expectation was tested as shown in Figure 7. We followed the production of active thrombin by noting the appearance of the proteolysis product prethrombin 1, and found that native prothrombin was activated to active thrombin at comparable rates with and without 0.1 mM DTT treatment. Thus, DTT did not significantly alter the global structure of prothrombin at this low concentration. This

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K_d = 4.4 \times 10^{-8} \text{ M}, \quad n = 1.00 \text{ mol of DAPA per mole of native thrombin}, \quad K_d = 1.7 \times 10^{-8} \text{ M [equation (1) in the paper by Pei et al. (1991)]}
\]
left us free to attempt removal of the chemical blockage on the engineered C-terminal Cys by mild reduction of P(S528A,G581C) with up to 0.1 mM DTT.

**Specific labeling of P(S528A,G581C)**

To optimize the experimental conditions for specific labeling, limited reduction with DTT at concentrations < 0.1 mM was performed at several stages in the P(S528A,G581C) purification. From the data obtained, the conditions for limited reduction of P(S528A,G581C) were set as a molar ratio of DTT to protein of 1.4 after isolation of the protein from these conditions.

We determined the ratio of probe to protein in the labeled prothrombin as described. For four batches of labeled double mutant prothrombin, the probe–protein stoichiometry was in a range from 0.8 to 0.95.

To determine if the structure of the labeled prothrombin [P(S528A,G581C)-FM] was similar to the unlabeled species, we activated prothrombin by prothrombinase and snake venoms. SDS–PAGE results (data not shown) showed that the activation rate for P(S528A,G581C)-FM by the fully assembled prothrombinase was 53% of the rate of activation of native prothrombin, compared with 55% for unlabeled P(S528A,G581C). Both activations proceeded to completion by comparable intermediates. The similarity between the activation kinetics of the labeled and the unlabeled double mutant prothrombin indicates that the structure of the double mutant prothrombin was not altered by the labeling procedure, making disulfide isomerization an unlikely complication during mild DTT reduction and fluorophore labeling.

In order to determine the location of the fluorescein label in P(S528A,G581C)-FM, we digested the labeled prothrombin with either native thrombin or Taipan venom. Prethrombin 1 and thrombin from P(S528A,G581C)-FM were fluorescent when viewed under UV light (Figure 9). These results demonstrate that the thio-specific fluorophore was located in the catalytic domain, which is the C-terminal part of the prothrombin molecule.

Carboxypeptidase Y, a C-terminal endopeptidase, was used to determine whether P(S528A,G581C)-FM was specifically labeled on the cysteine at the penultimate residue. Samples of P(S528A,G581C)-FM, P(S528A) and native prothrombin were incubated with carboxypeptidase Y, and the incubation mixtures were examined by SDS–PAGE at various time intervals. The results, shown in Figure 10, indicated that removal of the C-terminal amino acids from P(S528A,G581C)-FM was essentially blocked relative to results with native prothrombin or with P(S528A). Bands equivalent to prothrombin minus the last 12 or 24 residues (P-12, P-24) were seen with digestion of P(S528A) or native prothrombin, but were absent in the digestion of P(S528A,G581C)-FM. The sequence for the last 25 amino acids in the native or single mutant prothrombin is .GKYGPYTHVPRLKKWIQKVIDRGLS, with lysine at the twelfth and thirteenth positions from the C-terminus and lysine and glycine at the twenty-fourth and twenty-fifth positions from the C-terminus. Since the removal of lysine or glycine by carboxypeptidase Y is much slower than removal of other amino acids (Hayashi, 1976), the build-up of the P-12 and P-24 fragments is expected. The failure to detect the P-12
or P-24 fragments for P(S528A,G581C)-FM suggested that carboxypeptidase action was blocked at a residue closer to the C-terminus, i.e. in the last 12 amino acids. Comparing the sequence of the last 12 amino acids between P(S528A,G581C) and native prothrombin or P(S528A), the only difference is the cysteine replacing the glycine at the penultimate residue of P(S528A,G581C). Since both native prothrombin and P(S528A) were treated by DTT and FM in the same way as P(S528A,G581C) before the carboxypeptidase Y digestion, we conclude that the fluorescent probe is linked to the engineered cysteine, the penultimate residue of the labeled prothrombin. The only other possible explanation for our results is that the mutated cysteine is paired via disulfide isomerization with one of the buried cysteines in prothrombin and the rightful partner of this cysteine is labeled with FM. Although we cannot rule out this possibility for a small fraction of our mutant prothrombin population, it seems unlikely for the majority of P(S528A,G581C)-FM molecules because both P(S528A, G581C) and P(S528A,G581C)-FM have the same functional properties, and only P(S528A,G581C) but not native prothrombin is labeled following mild reduction.

Conclusion
After mild reduction, P(S528A,G581C) but not native prothrombin is labeled by a thiol-specific fluorescent reagent. When activated, P(S528A) and P(S528A,G581C) bind a specific thrombin active site inhibitor with equal affinity. P(S528A), P(S528A,G581C) and P(S528A,G581C)-FM can be activated with comparable kinetics by prothrombinase. Digestion experiments make it very likely that P(S528A,G581C)-FM is specifically labeled at the cysteine residue engineered into this double mutant protein. We conclude that a bovine prothrombin double mutant has been cloned, expressed, purified and specifically labeled at a specific engineered cysteine residue at the C-terminus. This molecule lacked enzymatic activity because of a mutated active site but otherwise showed native-like behavior. It should be a very useful tool for elucidating the structural role of membranes during prothrombin activation to thrombin.

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References