Pregnancy Zone Protein, a Proteinase-Binding Macroglobulin. Interactions with Proteinases and Methylamine†

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Received May 19, 1989; Revised Manuscript Received July 14, 1989

ABSTRACT: Human pregnancy zone protein (PZP) is a major pregnancy-associated plasma protein, strongly related to α_2-macroglobulin (α_2M). Its properties and its reactions with a number of enzymes, particularly chymotrypsin, and with methylamine have been investigated. It is concluded that native PZP molecules are dimers of disulfide-bridged 180-kDa subunits and that proteinase binding results in covalent 1:1 (tetrameric)PZP–enzyme complexes. Native PZP is unstable, and storage should be avoided, but when kept unfrozen at 0 °C most PZP preparations stay native 1–3 months. The reaction of PZP with chymotrypsin involves (i) proteolysis of bait regions, (ii) cleavage of β-cysteinyl-γ-glutamyl thiol ester groups, (iii) some change of the conformation and quaternary structure of PZP, and (iv) the formation of covalent 1:1 chymotrypsin–PZP(tetramer) complexes in which chymotrypsin is active but shows less activity than free chymotrypsin. The emission spectra of intrinsic fluorescence show significant differences between the PZP–chymotrypsin complex and its native components, whereas no differences are observed between methylamine-reacted PZP and native PZP. Methylamine reacts with the β-cysteinyl-γ-glutamyl thiol ester groups of PZP in a second-order process with $k = (13.6 \pm 0.5)$ M^{-1} s^{-1}, pH 7.6, 25 °C. The reaction product is PZP(dimers); no PZP(tetramers) are formed. The proteinase-binding specificity of PZP is far more restricted than that of α_2M. Certain chymotrypsin-like and trypsin-like enzymes are bound much less efficiently than is chymotrypsin itself. It is suggested that one reason for this is the importance of the covalent binding of the enzyme to PZP, which is mainly determined by the content of lysine residues of the enzyme. Another reason is the importance of bait region cleavage, which is determined by the substrate specificity of the enzyme. PZP is analogous to α_2M, but also distinct in several respects. (i) The native molecule is a dimer. (ii) The trap mechanism is not at work. Only covalently bound, sterically inhibited proteinase–(tetramer)PZP complexes are formed. (iii) No apparent major change of conformation is triggered by cleavage of the β-cysteinyl-γ-glutamyl thiol esters alone. (iv) Change of conformation requires interaction with a proteinase.

Human pregnancy zone protein (PZP)[1] is a pregnancy-associated plasma protein, which recently has been shown to belong to the group of proteinase-binding macroglobulins (Sottrup-Jensen et al., 1984; Sand et al., 1985), the high molecular weight glycoproteins of which α_2-macroglobulin (α_2M) is the best known representative [see Sottrup-Jensen (1987) for a recent review].

PZP was first described by Smithies (1959). It is a trace protein in normal plasma (<10 mg/L) but may reach a concentration of 1 g/L during pregnancy (Von Schoultz, 1974; Folkersen et al., 1981; Carlsson et al., 1987). Its function is unknown, but a number of studies have dealt with a possible role of PZP as an immunosuppressant in pregnancy (Van Schoultz et al., 1973; Stimson, 1976), and it has been suggested that PZP mainly controls the activity of cellular proteinases released under conditions of increased cellular turnover, e.g., during pregnancy (Sand et al., 1985).

Early studies showed that PZP is an α_2-glycoprotein which contains 10–12% carbohydrate and is composed of 180-kDa protein subunits organized as disulfide-bridged dimers that are further noncovalently assembled into tetramers and may form aggregates (Cooper, 1963; Kueppers, 1969; Bohn, 1971; Von Schoultz & Stigbrand, 1974; Bohn & Winckler, 1976; Stimson & Farquharson, 1978). Recently, approximately 50% of the amino acid sequence of PZP was determined, and its homology to that of α_2M was demonstrated (Sottrup-Jensen et al., 1984). Sand et al. (1985) showed that PZP is indeed a proteinase-binding protein and that the interaction of PZP with proteinases involves specific proteolytic cleavage of a critical peptide segment (the bait region) and cleavage of internal β-cysteinyl-γ-glutamyl thiol esters formed from side chains four residues apart in the polypeptide chains. This results in exposure of thiol groups and covalent binding of the proteinase to PZP. These events are analogous to those known from α_2M–proteinase interactions. In this paper, we present the results of studies which further elucidate structural and functional properties of PZP and of PZP–enzyme complexes.

Experimental Procedures

Materials. PZP was prepared from late-pregnancy plasma or serum as described by Sand et al. (1985). Freshly prepared

† This work was supported by grants from the Danish Science Research Council [Grants 11-6876 (U.C.) and 511-1079 (N.H.)] and from the Danish Cancer Society, the NOVO Foundation, and the Center for Eucaryotic Gene Regulation, University of Aarhus (L.S.-J.).

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[1] Abbreviations: PZP, pregnancy zone protein; α_2M, α_2-macroglobulin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; SGPA, *Streptomyces griseus* protease A; SGPB, *Streptomyces griseus* protease B; Suc-Ala-Ala-Pro-Phe-*p*NA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide; D-Val-L-Leu-L-Arg-*p*NA, D-valyl-L-leucyl-L-arginine 4-nitroanilide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate.

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PZP in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0 °C, contained only small amounts of 720-kDa tetramers and no aggregates of high molecular mass as judged from nondenaturing PAGE and from absorbance measurements at 280 nm [$E^{1\%}_{1cm}$ = 8.2 (Sand et al., 1985)] before and after filtering through 0.47-μm membranefilters. Aggregation inevitably occurred when PZP preparations were stored, and batches containing aggregated material gave irreproducible results. Of the three sets of storage conditions investigated, (i) at −20 °C, (ii) refrigerated at 5–8 °C, and (iii) kept in wet ice at 0 °C, the last was preferred. Typically, PZP preparations stored at 0 °C were stable for 1–3 months.

Bovine α-chymotrypsin was from BDH, Poole, U.K. Stock solutions (10–100 μM) in 10^{-3} M HCl, pH 3, were kept in aliquots at −20 °C. Their active-site concentrations were determined by titrations with soybean trypsin inhibitor (BHD, Poole, U.K.), the active concentration of which was determined by using 4-nitrophenyl 4-guanidinobenzoate titrated trypsin, a method analogous to that described by Ganrot (1967).

Streptomyces griseus proteases A and B (SGPA and SGPB) were prepared from Pronase (Sigma Chemical Co., St. Louis, Mo) essentially as described by Narahashi (1970). Their concentrations were calculated from rate determinations using Suc-Ala-Ala-Pro-Phe-*p*NA as substrate and the kinetic parameters determined by Christensen et al. (1985).

Bovine trypsin "Crystalline Trypsin Novo" was a gift from Novo (Copenhagen, Denmark). Plasmin was prepared as described by Christensen and Ipsen (1979). ^{125}I-Labeled PZP and ^{125}I-labeled chymotrypsin [approximately 90% active, determined by reaction with α_2M (Sand et al., 1985)] were prepared by the chloramine-T procedure using $Na^{125}I$ from Amersham, Buckinghamshire, U.K. (Hunter & Greenwood, 1962). Suc-Ala-Ala-Pro-Phe-*p*NA was from Bachem, Bubendorf, Switzerland. DTNB, methylamine, and other chemicals were analytical grade from Fluka, Buchs, Switzerland, or from Merck, Darmstadt, FRG.

Methods. To examine the dimer–tetramer distribution of PZP complexes, samples containing PZP [2.8 μM, approximately (based on a molecular mass of 720 kDa)] were incubated with 0–1.63 mol of ^{125}I-labeled chymotrypsin per mole of (tetramer)PZP in a total volume of 120 μL. After 2 min, 2 μL of 1 M DFP was added, and 10 μL was mixed with 50 μL of 10% v/v glycerol. Aliquots were subjected to nondenaturing PAGE in 5% gels, performed as described by Van Leuven (1981), and to SDS–PAGE (reduced and nonreduced). The distribution of the ^{125}I label in the gels was examined by γ counting in an LKB–Ultragamma counter. Methylamine-treated PZP samples were incubated for 15 min with 0.5 M methylamine, pH 7.6.

The extent of covalent binding of chymotrypsin to PZP was determined essentially as described by Sottrup-Jensen and Birkedal-Hansen (1989). Reaction mixtures containing PZP (2.75 μM tetramer) and ^{125}I-labeled chymotrypsin [0.22 mol/mol of (tetramer)PZP] in 0.01 M Na_2HPO_4/NaH_2PO_4 and 0.15 M NaCl, pH 7.5, were incubated at 22 °C for 5 min in the absence or presence of added nucleophiles (0.18 M methylamine or 0.09 M hydroxylamine) and were then made 10 mM in DFP and iodoacetamide. The ^{125}I-labeled chymotrypsin–PZP complexes were isolated by using Superose 12 gel chromatography eluted with the buffer. Then the complexes were denatured (50% v/v formic acid, 1 h), and the material was rechromatographed using 50% v/v formic acid as eluent. The amount (percent) of covalently bound chymotrypsin was calculated from the distribution of the ^{125}I label found.

The exposure of thiol groups in the reactions of PZP with enzymes and with methylamine was followed by measuring the change in absorbance at 412 nm using a Perkin-Elmer Lambda 17 UV–vis spectrophotometer in the presence of a large excess of DTNB at 25 °C essentially as described for α_2M–proteinase reactions by Christensen and Sottrup-Jensen (1984).

The kinetic parameters for hydrolysis of peptide substrates by the enzymes and the enzyme–PZP and enzyme–α_2M complexes were determined essentially as described by Christensen (1975). Titrations of PZP and α_2M using the change of kinetics of the enzyme when bound to the macroglobulin in the presence and absence of STI were performed essentially as described by Christensen and Sottrup-Jensen (1983).

Bait region cleavage of ^{125}I-labeled PZP by chymotrypsin [0–3 mol/mol of (tetrameric)PZP] was estimated from γ counting of the bands after PAGE separation of reduced and SDS-treated samples (Christensen & Sottrup-Jensen, 1984).

Fluorescence emission spectra were recorded in a Perkin-Elmer LS-50 fluorometer at 25 °C. The excitation wavelength was 280 nm, and the slits were 10 nm (excitation) and 4 nm (emission). All instrumental parameters were kept fixed during measurements, and each of the spectra illustrated in Figure 8 see averages of five scans. Inner filter effects were negligible. The experiments were performed at 0.9 mol of chymotrypsin per mole of (tetramer)PZP at PZP concentrations of 50 nM. Spectra were recorded of chymotrypsin, PZP, and mixtures thereof after incubation for 0–1 h, and of methylamine–PZP mixtures after approximately 1 h of reaction (50 nM PZP and 0.1 M methylamine hydrochloride in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer adjusted to pH 7.6 with NaOH).

The kinetics of the methylamine–PZP reaction were studied under pseudo-first-order conditions at eight (total) concentrations of methylamine in the range 0–320 mM and PZP 1.1 μM (tetramer). The reaction was followed by measuring the thiol group exposure as described above.

Results

Dimer–Tetramer Composition of Native PZP, of PZP-^{125}I-Labeled Chymotrypsin Complexes, and of CH_3NH_2-Treated PZP. The quaternary structures of the native and the chymotrypsin- and methylamine-treated forms of PZP were investigated by using nondenaturing PAGE (Figures 1A and 2). Figure 1 shows the autoradiogram and the Coomassie brilliant blue stained gel from samples containing 0–1.6 mol of ^{125}I-labeled chymotrypsin per mole of (tetramer)PZP. From Figure 1A, it is seen that *native* PZP exists primarily as 360-kDa dimers and that interaction of native PZP dimers with chymotrypsin results in the formation of complexes composed of PZP(tetramers) and chymotrypsin. As seen from Figure 2, no PZP(tetramers) appear when PZP is treated with methylamine.

During electrophoresis, a possible association/dissociation equilibrium shifts toward dissociation of the tetramers and toward association of the dimers as they separate. Electrophoresis thus can give misleading results for the oligomer state of the complexes of the sample applied.

Figure 3 shows the distribution of tetrameric and dimeric PZP–chymotrypsin complexes revealed from γ counting of the bands of Figure 1A. It is interesting that the stoichiometry is 1:1 mol of chymotrypsin/mol of PZP(tetramer) and that a binding ratio >1 is never observed. The chymotrypsin–PZP(dimer) complexes (Figure 1A) most probably stem from chymotrypsin–PZP(tetramer) complexes and are dissociation or degradation products thereof. If direct formation of final

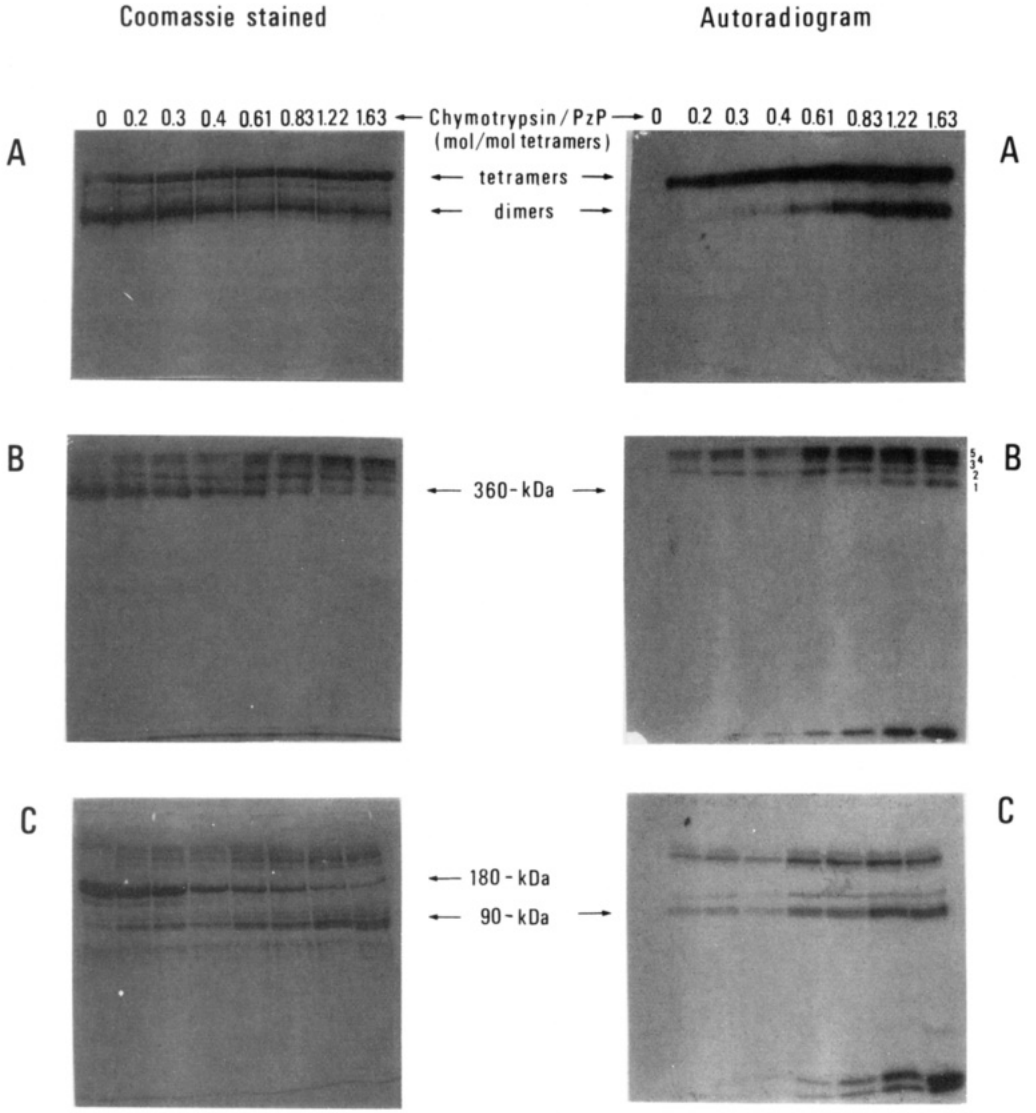

FIGURE 1: Titration of PZP with ^{125}I-labeled chymotrypsin. PAGE of PZP–^{125}I-labeled chymotrypsin reaction mixtures. (Right panels) Autoradiograms. (Left panels) Coomassie brilliant blue stained. (A) Nondenatured. (B) Denatured with SDS. (C) Reduced (dithioerythritol) and denatured (SDS). To reaction mixtures (120 μL) containing 0–1.6 mol of ^{125}I-labeled chymotrypsin/mol of PZP (based on tetramer) (2.8 μM) was added 2 μL of 1 M DFP after 2 min. Then 10 μL from each mixture was added to 50 μL of 10% glycerol; 3 × 15 μL was used for nondenaturing runs (65% gel) (A). Two of these gels were used to determine the amounts of ^{125}I-labeled chymotrypsin present in the protein bands (Figure 3). The rest (~15 μL) was mixed with 15 μL of SDS (2 w/v %) and heated to 95 °C for 5 min. From this mixture 5 μL was used for a denatured run (10% gel) (B). The remaining 25 μL was mixed with 5 μL of 0.2 M dithioerythritol and heated to 95 °C for 2 min, and from this mixture, 10 μL was used for a reduced, denatured run (10–20% gel) (C). α_2M was used as a standard.

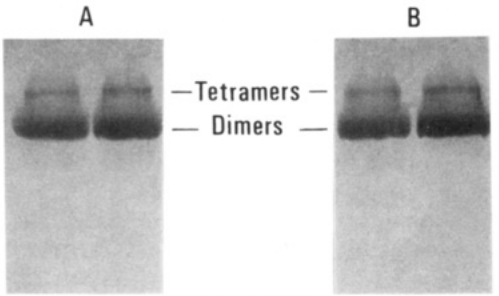

FIGURE 2: Nondenaturing PAGE of PZP and methylamine-reacted PZP. (A) Native PZP (2.4 μM) diluted 2.5-fold with 10% glycerol; 2 × 15 μL was applied to an 8% gel. (B) Mixture containing PZP (2.4 μM) and methylamine (0.5 M, pH 7.6) incubated 15 min, 25 °C, and then diluted 2.5-fold with 10% glycerol; 2 × 15 μL was applied to an 8% gel.

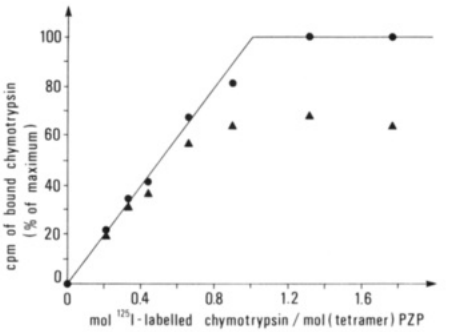

FIGURE 3: Amounts of ^{125}I-labeled chymotrypsin of the protein bands in Figure 1A. (●) Total amount of chymotrypsin in tetramer plus dimer bands. (▲) Amount of chymotrypsin in tetramer bands. 100% = total (=3029 cpm) at saturation of PZP with chymotrypsin; the results are the average of two determinations. For experimental details, see the legend to Figure 1.

chymotrypsin–PZP(dimer) complexes occurs, binding of up to 2 mol of enzyme per mole of PZP(tetramer) is expected. Further, no chymotrypsin–PZP(dimer) complexes are seen under circumstances where the concentration of chymotrypsin is low and PZP is in >2-fold excess. This indicates a correlation between the time-average concentration of free enzyme which governs the rate of nonspecific proteolysis and the

Table I: Covalent Binding of ^{125}I-Chymotrypsin to PZP[a]

presence of competing nucleophile	total binding [mol/mol of (tetramer)PZP][b]	% covalent binding[c]
	0.22	96
0.18 M methylamine	0.09	95
0.09 M hydroxylamine	0.03	95

[a] The results are the mean of two determinations (see Experimental Procedures). [b] Determined from the amount of chymotrypsin bound to PZP in nondenaturing conditions after gel chromatography. [c] Determined from the amount of chymotrypsin associated with PZP under denaturing conditions after gel chromatography (Sottrup-Jensen & Birkedal-Hansen, 1989).

formation of enzyme–PZP(dimer) complexes.

Figure 1B (nonreduced SDS–PAGE) shows the conversion of native PZP dimers to species of very high apparent molecular size after reaction with ^{125}I-labeled chymotrypsin which has been covalently bound. The bands labeled 1–5 presumably correspond to those discussed by Feinmann et al. (1985) of the α_2M–thrombin system. When comparisons are made with Figure 1A, it is seen that bands 2, 4, and 5 of Figure 1B are characteristic of tetrameric PZP–chymotrypsin complexes and bands 1 and 3 of dimeric PZP–chymotrypsin complexes.

Figure 1C (reduced SDS–PAGE) shows the pattern of cleavage of PZP. The (approximate) 90-kDa fragments resulting from bait region cleavages are prominent, but also nonspecific proteolysis occurs, particularly when chymotrypsin is in excess. The autoradiogram of Figure 1C reveals ^{125}I-labeled chymotrypsin covalently bound to one 90-kDa fragment of PZP, as well as to two such fragments or perhaps a 180-kDa PZP subunit. The pattern is similar to that of α_2M–proteinase complexes (Wang et al., 1983). The weakly labeled bands just above each of the main bands may correspond to reaction products of PZP with small amounts of π-chymotrypsin.

From experiments similar to those of Figure 1C, using ^{125}I-labeled PZP, the stoichiometry of moles of bait regions cleaved per mole of chymotrypsin bound was found to be approximately 4:1 (results not shown).

Reduced SDS–PAGE patterns of cleavage of PZP by equimolar amounts of chymotrypsin and of two microbial chymotrypsin-like enzymes, SGPA and SGPB, were performed after incubation for 5–15 min. PZP was rapidly degraded by the latter enymes (SGPB > SGPA). The same is seen only after long-term incubation with chymotrypsin (results not shown).

Covalent Binding of Chymotrypsin to PZP. The extent of covalent binding in the absence and presence of nucleophiles is shown in Table I. In contrast to human α_2M (Salvesen et al., 1981; Sottrup-Jensen et al., 1981), PZP clearly is not able to noncovalently trap a proteinase. When the enzyme and a nucleophile are added simultaneously and are competing for the thiol esters, only small amounts of proteinase–PZP complexes are formed. The same has been observed for the proteinase-binding macroglobulin rat α_1-inhibitor 3, which is monomeric in the native form (results not shown).

Thiol Group Exposure in Reactions of PZP with Enzymes. Figure 4 shows the concentration of thiol groups exposed during the reaction of chymotrypsin with PZP in a series of reaction mixtures containing various concentrations of chymotrypsin and a fixed concentration of PZP. In the molar range 0–1 chymotrypsin per PZP(tetramer), the stoichiometry (moles of thiol groups exposed to moles of chymotrypsin) is 4:1. Chymotrypsin bound in the resultant 1:1 mol of chymotrypsin/mol of (tetramer)PZP complex(es) clearly does *not* attack residual free PZP. If that was so, not only an amount

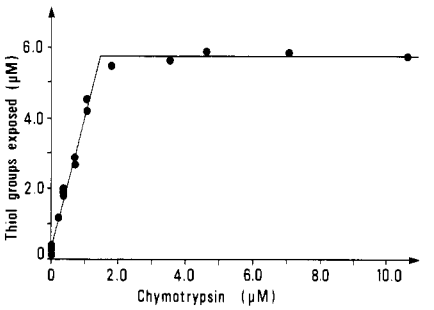

FIGURE 4: Titration of PZP thiol groups. The exposure of thiol groups was determined from measurements of the absorbance at 412 nm of reaction mixtures containing 0–10.6 μM chymotrypsin and 1.45 μM (tetramer) fresh, native PZP in the presence of 0.4 mM DTNB. The reactions were followed for 90 min. No changes in absorbance were seen after 10 min.

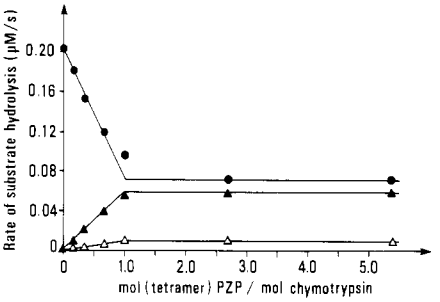

FIGURE 5: Enzymic activity of chymotrypsin and PZP-bound chymotrypsin. Initial velocity of hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*NA, pH 7.6, 25 °C, is shown. Rate assays performed after 45, 90, and 180 min of preincubation of chymotrypsin (24 nM) and PZP [0–124 nM (tetramer)] gave identical results within experimental error (1%). The average of such values is shown. Rate assays were performed at eight substrate concentrations [12.5–542.8 μM (138 μM in the series illustrated)] and total chymotrypsin (12 nM) in the absence (●) or presence of 1.5 μM STI (▲) 60 s and (△) 90, 150, and 300 min after addition of STI.

of thiol groups stoichiometrically related to that of chymotrypsin, but all of the thiol groups of the PZP present would appear eventually.

A series of experiments similar to those illustrated in Figure 4 were performed using trypsin, plasmin, SGPA, and SGPB. These experiments showed slow exposure of all of the PZP thiol groups. No stoichiometric relations between the enzyme concentrations and that of the thiol groups were observed (results not shown). In these cases, cleavage of thiol esters may at least partly be due to nonspecific reactions with solvent triggered by extensive proeolytic degradation of PZP.

Binding of trypsin and plasmin to PZP apparently is less efficient than that of chymotrypsin. Sand et al. (1985) observed a binding ratio of 0.4 mol/mol of (tetramer)PZP of trypsin and plasmin.

Enzymic Properties of PZP-Bound Proteinases. Figure 5 illustrates the effect on the enzymic activity of chymotrypsin after reaction with varying amounts of PZP. The hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*NA catalyzed by equilibrium mixtures of chymotrypsin and PZP–chymotrypsin complexes was investigated. One series of initial rates obtained at one of the substrate concentrations used is illustrated. In accordance with the results shown in Figure 3, the stoichiometry is 1:1 mol of chymotrypsin/mol of (tetramer)PZP. The rates decrease linearly with the concentration of tetrameric PZP until the equivalence point (1:1) is reached. At higher concentrations of PZP, where no free chymotrypsin is present and PZP–chymotrypsin complexes are the only species with enzymic activity, the rates do not change when the composition of the reaction mixture is greatly changed (from 0 to 5 times excess

Table II: Kinetic Parameters of Chymotrypsin, PZP-Bound Chymotrypsin, and α_2M-Bound Chymotrypsin Catalyzed Hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*Na[a]

enzyme species	K_m (μM)	k_c (s^{-1})	k_c/K_m (μM^{-1} s^{-1})
chymotrypsin	45 ± 3	22.0 ± 0.3	0.49 ± 0.04
PZP–chymotrypsin	66 ± 3	9.4 ± 0.2	0.14 ± 0.01
α_2M–chymotrypsin	105 ± 4	11.7 ± 0.2	0.11 ± 0.01

[a] Experimental conditions: 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.6, 25 °C.

of PZP based on tetrameric PZP). The kinetics of PZP-bound chymotrypsin indicate the presence of only one kind of PZP–chymotrypsin complex, the tetrameric PZP–chymotrypsin complex. If dimeric PZP–chymotrypsin exists, the enzyme thereof shows the same activity as that of the tetrameric PZP–chymotrypsin complex. The kinetic parameters are given in Table II.

The residual (PZP-protected) enzyme activity obtained at 60 s and >90 min (when equilibrium prevails) after addition of a great excess of STI to equilibrated PZP–chymotrypsin mixtures is also illustrated in Figure 5. All of the mixtures of STI, PZP, and chymotrypsin show the same percentage of the PZP-bound chymotrypsin activity at the same time, that is, 83 ± 1% (60 s) and at equilibrium (>90 min) 12 ± 0.5%. These results are in accordance with the covalent nature of the chymotrypsin–PZP complex.

The loss of activity *cannot* be due to dissociation of a reversible PZP–chymotrypsin complex with dissociation constant K in case K/K_D = [PZP][STI·C]/[PZP·C][STI], where the concentration of STI would be constant (1.5 μM), since the enzyme concentration is only 1% thereof. The ratio [PZP·C]/[STI·C], which is that of enzyme bound to PZP to enzyme bound to STI, would be proportional to the concentration of free PZP. No changes were observed over a 25-fold range of free [PZP].

We must conclude that ternary STI–chymotrypsin–PZP complexes form with the dissociation constant K_T = [STI]·[PZP·C]/[STI·C·PZP] = (0.12/0.88) 1.5 μM = 2.0×10^{-7} M. This value is only 13 times the K_D of the STI–chymotrypsin complex, which was determined to be 1.5×10^{-8} M (results not shown).

Similar experiments were performed to investigate the α_2M–chymotrypsin–STI interactions, and K_T = 1.5×10^{-6} M was obtained (results not illustrated). The difference in K_T values between α_2M- and PZP-bound chymotrypsin probably reflects more steric hindrance in the α_2M than in the PZP complex. The same tendency is apparent from the kinetic parameters on small synthetic substrates (Table II).

The kinetics of hydrolysis of some peptide-*p*-nitroanilide substrates of trypsin, plasmin, SGPA, and SGPB after their reaction with PZP were also investigated. For trypsin, plasmin, and SGPB, no changes of substrate kinetics were observed when followed for 180 min, and no enzyme activity was present after after 60 s of reaction with a great excess of STI (Figure 6). In contrast with chymotrypsin, these enzymes apparently form no sterically inhibited complexes with PZP. The binding of trypsin and plasmin to PZP is slow (Sand et al., 1985) and may compete poorly with the degradation of PZP by these enzymes. Figure 6 shows that the activity of SGPA is changed and partly protected against STI by PZP.

Fluorescence Emission Spectra. Figure 7 shows the uncorrected intrinsic fluorescence emission spectra of chymotrypsin, native PZP, and their complexes formed in reaction mixtures containing 0.9 mol of chymotrypsin per mole of (tetramer)PZP, 50 nM. Chymotrypsin (trace B) emits relatively weakly with a broad maximum located at 341 nm. The low intensity reflects the density of tryptophans, 7 per mole. The position of the maximum indicates a relatively high degree of exposure of the tryptophans to the aqueous medium. Hydrogen bonding is known to cause reduction in the fluorescence quantum yield (Lakowicz, 1983). PZP (trace A) emits with higher intensity at shorter wavelength (334 nm), reflecting a higher content of tryptophans (approximately 40 per mole), which on average are embedded in more hydrophobic regions of the protein.

Upon complex formation (trace D), the fluorescence intensity is increased, exceeding the sum of the emission from the uncomplexed components (trace C) by approximately 20%. Subtracting trace C from trace D results in a spectrum (trace E) with a maximum at 323 nm. Both observations imply that tryptophans from either PZP or chymotrypsin are shielded from the aquous medium in the complex (Lakowicz, 1983). Reaction with methylamine causes no change of the PZP fluorescence.

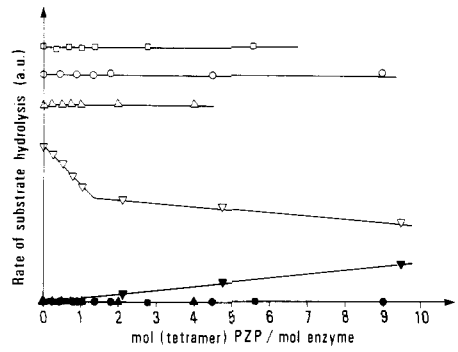

FIGURE 6: Enzymic activities of trypsin, plasmin, SGPA, and SGPB after incubation with PZP. The experiments were analogous to those illustrated in Figure 5. Open symbols show results obtained in the absence and closed symbols those obtained in the presence of STI for 60 s. D-Val-Leu-Arg-*p*NA was used as substrate for trypsin (□) and plasmin (○) and Suc-Ala-Ala-Pro-Phe-*p*NA as substrate for SGPA (▽) and SBPB (△). Addition of STI ($\sim 10^2 K_D$) totally inhibits PZP–trypsin (■), PZP–plasmin (●), and PZP–SGPB (▲), but not quite PZP–SGPA (▼) after 60 s even at 5–10-fold excess of PZP. a.u., arbitrary units.

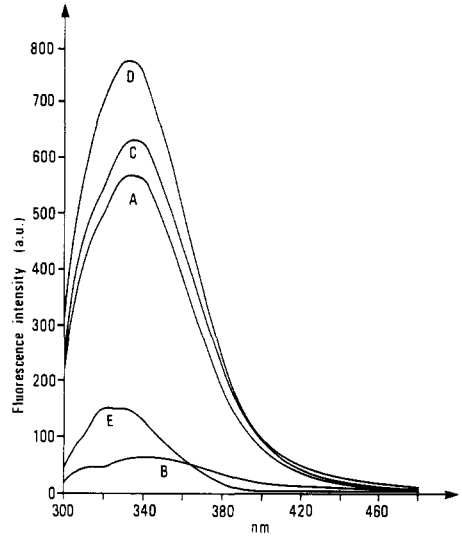

FIGURE 7: Uncorrected fluorescence spectra of PZP and chymotrypsin. All instrumental parameters were kept constant (A–E). All spectra are averaged from five scans. (A) PZP (50 nM). (B) Chymotrypsin (45 nM). (C) Sum of traces A and B. (D) Complex between PZP (50 nM) and chymotrypsin (45 nM). (E) Difference spectrum (trace D minus trace C). The spectrum of PZP (50 nM) in 1 M methylamine, HCl, pH 7.6 (incubation 1 h), was identical with trace A within experimental error.

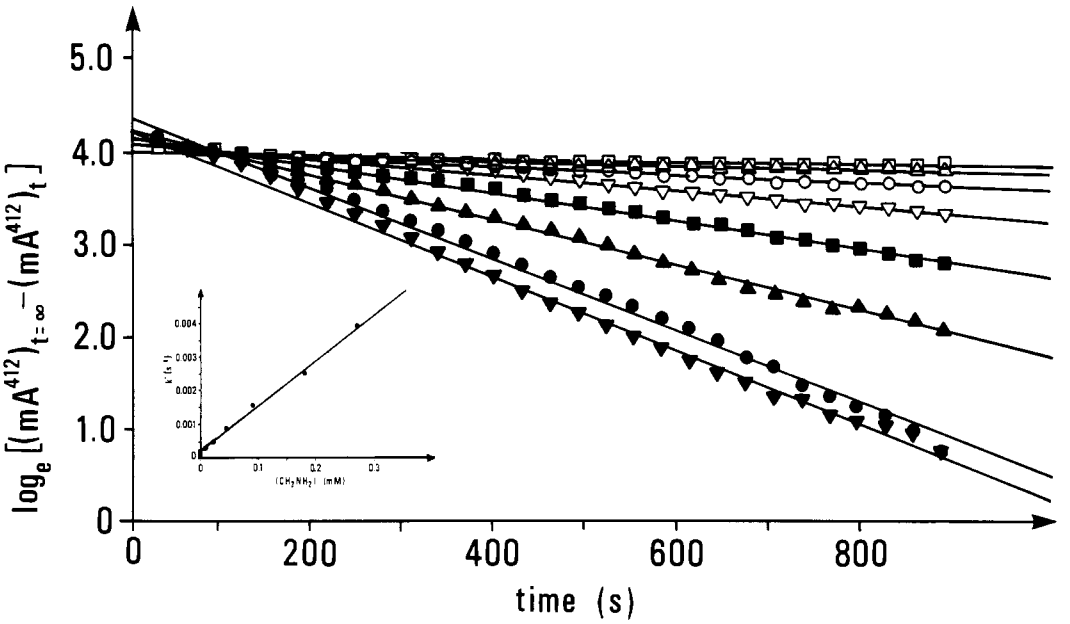

FIGURE 8: Pseudo-first-order kinetics of thiol group exposure in the reaction of PZP with methylamine. An excess of DTNB (0.4 mM) and PZP (1.1 μM) (tetramer) were mixed at time $t = 0$ with methylamine [(□) 0, (△) 8, (○) 20, (▽) 40, (■) 80, (▲) 160, (●) 240, and (▼) 320 mM total] at pH 7.6, 25 °C, and the reaction was followed by measuring the absorbance at 412 nm for 150 min ($t = \infty$). The absorbances are given in milliabsorbance units (mA). The lines shown are those obtained from least-squares fits. Insert: The pseudo-first-order rate constant, k', obtained from the slopes of the lines is plotted against the concentration of unprotonated methylamine ($pK_B = 3.45$). From $k' = k[CH_3NH_2]$, the second-order rate constant, $k = 13.6 \pm 0.5$ M^{-1} s^{-1}, is obtained (pH 7.6, 25 °C).

Kinetics of the Reactions of Methylamine with PZP and α_2M. These reactions were studied by measuring the exposure of thiol groups as a function of time at eight concentrations of $CH_3NH_2/CH_3NH_3^+$ in the range 0–0.32 M. Figure 8 illustrates the (pseudo) first-order behavior of these reactions and that a rectlinear dependence is obtained of the corresponding pseudo-first-order rate constant k' on the concentration of unprotonated methylamine calculated from the total concentration and pK_B. At the experimental conditions used, $pK_B = 3.45$ (Harned & Owen, 1930). Equivalent data were obtained on the α_2M reaction (not shown). The resulting values of the second-order rate constants of the PZP- and the α_2M–methylamine reactions were identical within experimental error:

$$k_{PZP} = 13.6 \pm 0.5 \text{ M}^{-1} \text{ s}^{-1}$$

$$k_{\alpha_2M} = 13.9 \pm 0.4 \text{ M}^{-1} \text{ s}^{-1}$$

The value of k_{α_2M} is in good agreement with that obtained by Larson and Björk (1984).

Discussion

PZP shares a number of characteristic features with α_2M and is a proteinase-binding macroglobulin (Sand et al., 1985). PZP has the same size of subunits (180 kDa) and is homologous with α_2M (Sottrup-Jensen et al., 1984). PZP molecules possess β-cysteinyl-γ-glutamyl thiol ester groups and form complexes with several proteolytic enzymes (e.g., chymotrypsin). The PZP-complexed enzyme is partly active, and the PZP subunits are proteolytically cleaved in two approximately 90-kDa protein fragments at a particular stretch of peptide called the bait region (Sottrup-Jensen et al., 1984, 1989; Sand et al., 1985). The sequence of the PZP bait region contains no lysyl residues and only one arginyl residue [in the sequence Arg-Pro (Sottrup-Jensen et al., 1989)]. Therefore, it is not expected to be cleaved easily by trypsin and trypsin-like enzymes (e.g., plasmin). The PZP bait region, however, contains residues meeting the substrate specificity of chymotrypsin and chymotrypsin-like enzymes (e.g., SGPA and SGPB) (Bauer, 1976, 1978). We have investigated the interactions of PZP with chymotrypsin, SGPA, SGPB, trypsin, and plasmin and studied the reaction of PZP with methylamine by means of protein band pattern analysis after PAGE, titration of thiol groups, measurements of enzyme activities, and fluorescence.

Of the enzymes investigated, chymotrypsin is the only one which forms complexes with PZP readily and with well-defined stoichometry. The results strongly indicate that the complexes are formed from the interaction of chymotrypsin with two molecules of native PZP(dimer), resulting in a 1:1 chymotrypsin–PZP(tetramer) complex (Figures 1, 3, 4, and 5). Four moles of thiol groups is titrated per mole of chymotrypsin added (Figure 4). Thus, all of the thiol esters of the four PZP subunits of the complex are cleaved apparently in concert with the cleavage of four bait regions. The enzymic activity of chymotrypsin is reduced, corresponding to the formation of 1:1 chymotrypsin–PZP(tetramer) complex(es) (Figure 5, Table II). Apart from the assembly of the PZP(dimers) into tetramers, the reaction of chymotrypsin with PZP displays features quite similar to those of the α_2M–proteinase reactions (Sottrup-Jensen, 1987).

However, the results also demonstrate great differences between PZP and α_2M. α_2M binds most kinds of proteinases, but PZP appears to be much more restrictive. If we assume as suggested for α_2M (Christensen & Sottrup-Jensen, 1984) that bait region cleavage is an essential trigger of the proteinase-binding reaction, it is quite understandable that neither trypsin nor plasmin readily forms PZP complexes. With Pro as the amino acid of the P_1' site of the only potential trypsin cleavage site (Sottrup-Jensen et al., 1989), the bait region is indeed expected to be a poor substrate of trypsin and plasmin (Christensen, unpublished results). Although slow secondary proteolysis may occur of chymotrypsin-sensitive sites in the bait region (Sottrup-Jensen et al., 1989), proteolysis outside that region is likely to be prominent. Such general cleavage of PZP competing with bait region cleavage probably explains the nonstoichiometric exposure of thiol groups and the kinetic

results obtained with plasmin and trypsin (Figure 6). Results of similar nature were obtained with SGPA and SGPB (Figure 6). These enzymes, however, are chymotrypsin-like enzymes and are expected to readily cleave at several sites of the bait region of PZP. It is likely, in view of the absence of lysyl residues in SGPA (Johnson & Smillie, 1974) and the presence of only one such residue in SGPB (Jurzerek et al., 1974), that largely no covalent complexes are formed, so that SGPA and SGPB are free to cleave all of the bait regions and furthermore cause extensive degradation of PZP.

1:1 Chymotrypsin–PZP(Tetramer) Complex. After nonreduced and reduced SDS–PAGE of ^{125}I-labeled chymotrypsin–PZP complexes, the label is found in protein bands representing covalently bound chymotrypsin (Figure 1). Gel chromatography experiments showed approximately 95% covalently bound PZP–chymotrypsin complexes (Table I), consistent with the result (Figure 5) that no dissociation is induced by a great excess of STI. The presence of nucleophiles during the PZP–chymotrypsin reaction greatly reduces the amount of chymotrypsin bound, but not the extent of covalent binding (Table I). In contrast to this, α_2M in similar conditions entraps a substantial amount of noncovalently bound enzyme (Sottrup-Jensen et al., 1981; Salvesen et al., 1981). The trap mechanism is apparently not active in PZP–proteinase binding.

The results of the kinetic and equilibria studies (Figure 5, Table II) indicate that steric hindrance of the access of solutes to chymotrypsin in the PZP complex is less than that in the α_2M complex. Measured as a difference in the standard free energy of complexation of chymotrypsin with the ligand, those of STI are $\Delta(\Delta G°) \sim 6.5$ kJ/mol (PZP) and $\Delta(\Delta G°) \sim 12$ kJ/mol (α_2M), whereas those of the peptide substrate are $\Delta(\Delta G°) \sim 0.1$ kJ/mol (PZP) and $\Delta(\Delta G°) \sim 0.2$ kJ/mol (α_2M) (from the K_m values).

The fluorescence spectra (Figure 7) also indicate a more solvent-accessible structure of PZP complexes compared with α_2M complexes. α_2M itself fluoresces at 332 nm, a marginal shift to the blue compared to native PZP (334 nm). This means that the tryptophans of the two native proteins on average are exposed to the solvent to the same extent. However, upon complexation with chymotrypsin, the fluorescence of PZP is enchanced only approximately half as much (~20%) as that of α_2M (~45%) (Björk & Fish, 1982). Apparently, reaction of the thiol esters with methylamine alone triggers the molecular changes giving rise to α_2M fluorescence changes (Björk & Fish, 1982). In contrast to α_2M, PZP shows no changes of fluorescence after reaction with methylamine. The methylamine reaction does not induce the association of PZP dimers into tetramers (Figure 2). The PZP fluorescence changes observed as a consequence of complexation with chymotrypsin thus may arise mainly from shielding of tryptophans of chymotrypsin and/or from the dimer–tetramer PZP association and not from major conformational changes within the subunit of PZP.

The reaction of methylamine with PZP shows simple second-order kinetics with a rate constant $k = 13.6 \pm 0.5$ M^{-1} s^{-1} (Figure 8). Exactly the same value is obtained with α_2M. The reactivity of the thiol esters thus is the same of the two proteins and is identical for each subunit, whether it is part of a dimer or a tetramer. The molecular forces of the subunit interactions apparently do not influence the thiol esters.

In summary, PZP shows many similarities to α_2M as well as marked differences. The restrictive proteinase-binding specificity of PZP is most striking. It results from two features: (i) the particular amino acid sequence of the bait region, that contains no peptide bonds at which fast cleavage by trypsin-like enzymes can take place; and (ii) the dependence on the internal thiol for covalent binding of the enzyme. Proteinases devoid in lysyl residues or possessing only a few of these are thus presumably prevented from efficient complex formation with PZP.

The conversion of native dimeric PZP to complexed tetrameric PZP is another striking feature of its reaction with proteinases. It suggests that PZP should not simply be considered as a "half tetrameric" α-macroglobulin. Its role in the control of proteinases in pregnancy is presently unknown.

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Autoactivation of Human Recombinant Coagulation Factor VII

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Received May 8, 1989; Revised Manuscript Received July 7, 1989

ABSTRACT: Single-chain human recombinant factor VII produced by transfected baby hamster kidney cells was purified to homogeneity in the presence of benzamidine. The amidolytic activity of single-chain recombinant factor VII with a peptidylnitroanilide substrate, methoxycarbonyl-D-cyclohexanylglycyl-L-arginine-*p*-nitroanilide, was less than 1% of that obtained with factor VII_a. Purified single-chain recombinant factor VII spontaneously activated in the absence of inhibitor. The activation reaction was enhanced by at least 2 orders of magnitude in the presence of a positively charged surface, provided either as an anion-exchange matrix or as poly(D-lysine). The progress curve for factor VII_a generation was sigmoidal. Benzamidine inhibits recombinant factor VII_a activity and factor VII activation with identical inhibition constants (K_i) of 11 mM. In contrast, benzamidine inhibition of bovine factor X_a and bovine factor II_a was observed at K_i values equal to 0.3 and 0.5 mM, respectively. Bovine factors X_a and II_a are known activators of factor VII and the most likely contaminants of our recombinant factor VII preparations. Single-chain recombinant factor VII purified from cells cultured in the absence of bovine serum activated at the same rate as factor VII from cells cultured in the presence of bovine serum. This also excluded the possibility that the activation reaction was caused by contaminating bovine proteases. On the basis of these observations, we propose that factor VII is autoactivated in vitro in the presence of a positively charged surface.

Coagulation factor VII (coagulation FVII)[1] is a vitamin K dependent protein playing a key role in the extrinsic pathway of blood coagulation. The protein belongs to the family of serine proteases. In its activated form, $FVII_a$, the protease catalyzes the activation of two other vitamin K dependent coagulation factors of the serine protease family, FIX and FX. Ultimately, this leads to the formation of a fibrin clot (Davie et al., 1979). As reported by Berkner et al. (1986), FVII can be produced in high yields by transfected baby hamster kidney cells. rFVII is secreted into the cell culture medium in its single-chain form just as the native molecule when synthesized in vivo by liver cells (Wion et al., 1985). Whether or not single-chain FVII is a genuine zymogen has been a matter of controversy. Using the bovine plasma derived FVII, Zur et al. came to the conclusion that FVII as well as $FVII_a$ possesses enzymatic activity (Zur et al., 1982). This conclusion has recently been challenged in studies of the human molecule (Rao et al., 1986; Rao & Rapaport, 1988). The answer to this question is of importance for an understanding of the initiation of the extrinsic pathway of coagulation. However, the problem has been hard to solve because the physiological substrates, FIX and FX, back-activate FVII once they are activated. Physiologically the activation of FVII seems to occur as a result of FIX_a (Seligsohn et al., 1979) or FX_a generation (Rao et al., 1986); however, in vitro experiments have shown that activation of FVII can also be accomplished by $FXII_a$ (Broze & Majerus, 1980) or by FII_a (Radcliffe & Nemerson, 1975). During purification of the plasma-derived bovine protein (Radcliffe & Nemerson, 1975) and the human recombinant protein (Thim et al., 1988), FVII was activated into the two-chain form by hydrolysis of the Arg_{152}–Ile_{153} bond. In both cases, the activation occurred on an anion-exchange column. Radcliffe and Nemerson (1975) suggested that activation of the bovine protein could be due to contamination with another bovine coagulation enzyme. This could also be true for the recombinant protein since the cell culture medium contains bovine serum. To investigate this possibility, we purified single-chain rFVII from serum-free cell culture medium and compared the activation on the anion matrix of this purified protein to the activation of rFVII obtained from serum-containing cell culture medium. The effect on the activation reaction of various inhibitors was also tested. Furthermore, these preparations were used to study the kinetics of FVII activation. Finally, the rFVII preparations were used to answer the question whether rFVII relative to $rFVII_a$ possesses significant activity with a peptidylanilide substrate.

[1] Abbreviations: FVII, coagulation factor VII; rFVII, recombinant FVII; FIX, factor IX; FX, factor X; FII, factor II; FXII, factor XII; SBTI, soybean trypsin inhibitor; RVV, Russel's viper venom; CH_3OCO-D-CHA-Gly-Arg-*p*NA, methoxycarbonyl-D-cyclohexanylglycyl-L-arginine-*p*-nitroanilide hydroacetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Q FF, Q Sepharose Fast Flow.

Experimental Procedures

Materials

Q and S Sepharose Fast Flow, CNBr-activated Sepharose 4B, PD-10 gel filtration columns, and low molecular weight

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