Human Tissue Factor Contains Thioester-Linked Palmitate and Stearate on the Cytoplasmic Half-Cystine†

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ABSTRACT: The state of the five half-cystine residues in human tissue factor (TF) has been characterized. The results indicate that the four half-cystines in the extracellular domain of TF form two disulfide bonds and the half-cystine in the cytoplasmic region is acylated by palmitic acid and stearic acid. The extracellular disulfide cross-links, Cys^{49}–Cys^{57} and Cys^{186}–Cys^{209}, were deduced from the analysis of tryptic peptides. Acylation of the cytoplasmic half-cystine was demonstrated by purifying and characterizing fibroblast TF from cells labeled with [^{3}H]palmitic acid. Radiolabeled fibroblast TF was observed by autoradiography following sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The tritiated material covalently bound to the protein was identified as [^{3}H]palmitate and [^{3}H]stearate by reverse-phase high-pressure liquid chromatography. Deacylation of TF with hydroxylamine resulted in the spontaneous generation of disulfide-linked TF dimers. This result suggests that the disulfide-linked TF dimer, a minor component of most TF preparations, and the recently described heterodimeric form of TF are artifacts produced by deacylation of Cys^{245} and subsequent interchain disulfide bond formation.

When blood contacts damaged tissue, coagulation is initiated by a complex formed of tissue factor (TF),[1] an integral membrane protein, and factor VII, a plasma protein. The cDNA coding for human TF has been isolated and sequenced as has much of the mature protein (Spicer et al., 1987; Morrissey et al., 1987; Scarpati et al., 1987; Fisher et al., 1987). The molecule contains five half-cystine residues of which only four can be involved in intrachain disulfide bonds. In this paper we assign the disulfides to the four half-cystines on the extracellular face of the molecule.

The state of the half-cystine residue in the cytoplasmic domain is unknown. Human TF has been reported to be principally a monomeric protein with traces of disulfide-linked dimer (Broze et al., 1985; Guha et al., 1986; Carson et al., 1987). Recently, however, two groups have identified a disulfide-bonded heterodimeric form of the molecule (Morrissey et al., 1987; Carson, 1987; Carson et al., 1988).

We have addressed the question of the relationship between the single-chain and multimeric forms of human TF by characterizing the cytoplasmic half-cystine. Evidence is presented demonstrating that this residue is acylated by palmitic and stearic acids. We conclude that the dimeric forms of the molecule are most likely purification artifacts produced by deacylation and subsequent interchain disulfide bond formation.

Materials and Methods

TF Isolation and Iodoacetamide Labeling. Human brain TF was purified by immunoaffinity chromatography as previously described (Carson et al., 1987; Spicer et al., 1987). The isolated protein, ≃100 μg/mL in 0.1 M NaCl and 0.05 M Tris, pH 7.5 (TBS), containing 0.1% Triton X-100, was precipitated by addition of trichloroacetic acid to give 10% (w/v). After incubation on ice for 15 min and centrifugation at 5000*g* for 30 min at 4 °C, the supernatant was discarded and the pellet extracted with acetone. The protein was then dissolved in 6 M guanidine hydrochloride containing 0.5 M Tris and 2 mM EDTA, adjusted to pH 8.1. The solution (≃500 μg of TF/mL) was divided in half and $^1/_{50}$ volume of dithiothreitol, 45 mM in H_2O, was added to one tube. Both samples were incubated at 50 °C for 30 min under N_2, and $^1/_{50}$ volume of 100 mM [^{14}C]iodoacetamide in H_2O (New England Nuclear, 24.1 mCi/mmol) was added to each. After 30 min in the dark at 24 °C under N_2, Triton X-100 was added to give a final concentration of 0.1%, and the samples were dialyzed against TBS–0.1% Triton X-100 at 4 °C until no radioactivity was detected in the buffer. Amino acid analysis of the labeled samples was performed as described elsewhere (Carson et al., 1987). The ^{14}C content was assessed by liquid scintillation counting using 10 mL of Opti-Fluor (Packard). Counting efficiency was estimated with a ^{14}C standard of predetermined specific radioactivity.

In Situ Labeling, Purification, Autoradiography, and Western Blot Analysis of Fibroblast TF. Fetal human lung fibroblasts (GM 1604, NIGMS Human Genetic Mutant Cell Repository) were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Hyclone, defined serum), penicillin (200 units/mL), and streptomycin (200 μg/mL). The cells were grown at 37 °C in a CO_2 incubator and were utilized from passage 8 to passage 11. Prior to radiolabeling, confluent fibroblast monolayers were growth-arrested by changing to medium containing 0.25% fetal calf serum. As with WISH amnion cells (Maynard et al., 1976) serum starvation reduced the TF content of the fibroblasts (data not

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[1] Abbreviations: TF, tissue factor; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; TBS, 0.1 M NaCl and 0.05 M Tris, pH 7.5; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; PC, phosphatidylcholine; PS, phoshatidylserine; Cys, half-cystine; CoA, coenzyme A; kDa, kilodalton(s); cpm, counts per minute.

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shown). After 48 h in low serum, TF synthesis was induced by returning the cells to medium containing 10% fetal calf serum. Four hours later 0.5 mCi of [^{3}H]palmitic acid (New England Nuclear, 28.5 Ci/mmol) in 10 mL of the same medium was added to each 75-cm^2 culture flask. After 8 h the cells were rinsed 3 times with 10 mL of Hank's balanced salt solution without divalent cations and were immediately scraped into TBS–1% Triton X-100 (5 mL/flask). The material was then centrifuged at 15000g for 15 min at 4 °C. Solubilized TF in the supernatant was immunopurified as before. Elution of TF procoagulant activity from the column was monitored by a two-stage clotting assay following relipidation (Bach et al., 1981). The TF peak was dialyzed against TBS–0.1% Triton X-100 and concentrated with an Amicon YM10 filter. The material was stored at −80 °C prior to analysis. Samples were precipitated and acetone extracted as above. SDS–PAGE was performed with 10% polyacrylamide gels (Laemmli, 1970). The protein pellets were dissolved and denatured at 80 °C for 5 min in sample buffer, with or without 5% 2-mercaptoethanol as indicated. Autoradiography was performed with Enlightning (New England Nuclear) according to the manufacturer's instructions. The film, Kodak X-Omat AR, was exposed for 10 days at −80 °C. The brain TF autoradiography standard was radiolabeled following the procedure previously employed for bovine TF (Bach et al., 1986). Western blot analysis was performed as described elsewhere (Carson et al., 1987) with a monoclonal antibody (10 μg/mL) against human TF.

HPLC Analysis of the Radiolabeled Fatty Acids. Pure TF from cells fed radiolabeled palmitic acid was isolated from two 75-cm^2 flasks. The protein, in 100 μL of TBS–0.1% Triton X-100, was precipitated with trichloroacetic acid and extracted with acetone (2 × 1 mL) followed by chloroform/methanol (2:1) until tritium was no longer detected in the organic solvent (5 × 1 mL). Five hundred micrograms of palmitic and stearic acids was added, and the sample was hydrolyzed in vacuo in 6 N HCl for 16 h at 115 °C. The hydrolysate was dried in a vacuum centrifuge and subsequently extracted 5 times with 200 μL of benzene. The benzene extract was evaporated under vacuum, and the material was esterified with methanolic HCl (Alltech Associates, Inc.) following the supplier's instructions. After drying, the methylated sample was dissolved in 250 μL of methanol; 10% was saved for liquid scintillation counting, and the remainder was characterized by reverse-phase HPLC using a Waters μBondapak C_{18} column (3.9 mm × 30 cm in 80% acetonitrile and 20% H_2O). The flow rate was 1 mL/min and 1-mL fractions were collected. Liquid scintillation counting was performed as described above.

Hydroxylamine Deacylation. Human brain TF (≃500 μg/mL) in TBS–0.1% Triton X-100 was dialyzed at room temperature for 20 h against 1 M hydroxylamine adjusted to pH 6 with NaOH. The protein was then dialyzed back into the starting buffer. CNBr cleavage was performed as previously described except that the succinic anhydride step was omitted (Spicer et al., 1987).

Reconstitution and Functional Assay. Deacylated and untreated brain TFs were reconstituted into phospholipid vesicles containing pure phosphatidylcholine as well as 30% phosphatidylserine (PS) and 70% phosphatidylcholine (PC) as previously described (Bach et al., 1986) except that the gel filtration step was omitted. The protein concentrations prior to relipidation were determined by amino acid analysis. The procoagulant activity of the relipidated material was determined in the two-stage clotting assay (see above) calibrated with a sample of ^{3}H human brain TF reconstituted in PS/PC (30:70) vesicles. The plot of log [clot time (s)] vs log [[^{3}H]TF (pg/mL)] was linear for the standard from 200 to 5 pg/mL [log [[^{3}H]TF (pg/mL)] = −2.33 log [clot time (s)] + 4.69; R^2 = 0.999].

Results and Discussion

Human TF is a single polypeptide chain containing 263 amino acids. From the sequence it is evident that the protein is composed of three distinct domains. The first 219 residues are on the extracellular side of the plasma membrane followed by 23 membrane-spanning hydrophobic amino acids and a 21-residue cytoplasmic region. There are five half-cystine residues: four in the extracellular domain and one on the cytoplasmic tail. The state of the sulfur in these half-cystines has not been determined. The question is important since the answer would resolve a controversy that has arisen about the state of TF in vivo. The initial reports described purified TF as a monomer with a small (<10%) amount of TF dimer (Broze et al., 1985; Guha et al., 1986; Carson et al., 1987). However, with the identification of the disulfide-linked heterodimeric form of the protein (Morrissey et al., 1987; Carson, 1987; Carson et al., 1988) there is as yet no consensus regarding what constitutes native TF.

The following evidence suggests that the four half-cystines in the extracellular domain of TF form two disulfide bonds, while the fifth is sequestered inside the cell. This segregation was shown by cleaving the protein, which contains a single methionine, with CNBr and using SDS–PAGE without reduction to separate residues 1–210, containing all the extracellular half-cystines, from residues 211–263, containing the cytoplasmic Cys^{245} (Spicer et al., 1987). In addition, we have separated the unreduced CNBr fragments by HPLC gel filtration in SDS (Takagi, 1981). The identities of the two peptides were confirmed by analytical SDS–PAGE, amino acid composition, and NH_2-terminal sequence analysis (data not shown). From these data it is evident that Cys^{245} is not covalently coupled to any of the extracellular half-cystines.

Human TF was tested for free sulfhydryl groups by allowing the denatured protein to react with [^{14}C]iodoacetamide as described under Materials and Methods. The unreduced, denatured TF incorporated only 0.049 mol of radioactive label/mol of TF, indicating that unreduced TF has no free sulfhydryl groups. When the denatured protein was reduced with dithiothreitol prior to labeling, 4.78 mol of [^{14}C]iodoacetamide was incorporated per mole of protein. Amino acid analysis gave 4.64 (carboxymethyl)cysteine residues per TF, consistent with the five SH groups expected to be generated by reduction.

The low level of [^{14}C]iodoacetamide incorporation into the extracellular half-cystines of unreduced TF can most easily be explained by the presence of disulfide bridges. Thus, we determined the location of these linkages by isolation and characterization of peptides obtained after tryptic digestion of unreduced TF (Spicer et al., 1987). The first two half-cystines, 49 and 57, were found in three separate but overlapping peptides that comprised residues 49–65, 47–65, and 47–68, respectively. The positions of the half-cystine residues were assigned by noting the absence of a phenylthiohydantoin amino acid derivative at the appropriate cycle of sequencing. In each peptide there was a blank at positions 49 and 57. Cys^{209} was identified in the tryptic peptide comprising residues 202–214, and Cys^{186} was found in a peptide containing residues 170–196. Both peptides coeluted from the reverse-phase column. On the basis of the retention times predicted (Guo et al., 1986), copurification of these two peptides in the absence of a covalent linkage is highly unlikely. From these results

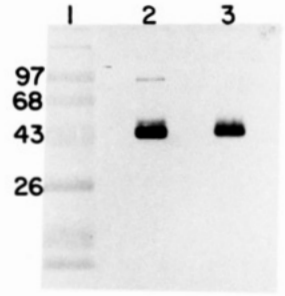

FIGURE 1: Western blot of brain and fibroblast TF. SDS–PAGE and Western blotting were performed as described under Materials and Methods. The lane assignments are as follows: (1) BRL prestained high molecular weight standards (phosphorylase *b*, 96 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 26 kDa); (2) 100 ng of human brain TF; (3) human fibroblast TF purified from one 75-cm^2 culture flask. The protein standards were reduced with 2-mercaptoethanol while the samples in lanes 2 and 3 were unreduced. The blot was developed with a monoclonal antibody against human TF.

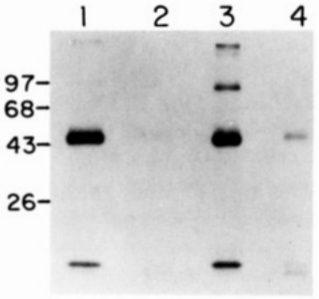

FIGURE 2: Autoradiogram of fibroblast TF labeled in situ with [^{3}H]palmitic acid. SDS–PAGE and autoradiography were performed as described under Materials and Methods. Lanes 1 and 3 contain ^{3}H human brain TF standard (5000 cpm per lane). Lanes 2 and 4 each contain human fibroblast TF purified from two 75-cm^2 culture flasks. The samples in lanes 1 and 2 were reduced with 2-mercaptoethanol, and in lanes 3 and 4 they were unreduced.

we conclude that the disulfide links are Cys^{49}–Cys^{57} and Cys^{186}–Cys^{209}.

The evidence described above suggests that the cytoplasmic half-cystine in TF does not contain a free sulfhydryl side chain and does not form an intrachain disulfide bond. The coupling of palmitic acid to cytoplasmic half-cystines in several membrane proteins has been reported (Schmidt & Schlesinger, 1979; Omary & Trowbridge, 1981; O'Brien & Zatz, 1984; Olson et al., 1984; Kaufman et al., 1984; Dolci & Palade, 1985; Koch & Hammerling, 1986; Hedo et al., 1987; Sefton & Buss, 1987). These findings prompted us to consider the possibility that TF is acylated in the same way. To test this, we labeled human fibroblasts in situ with [^{3}H]palmitic acid. Fibroblast TF was then extracted from the cells and immunopurified as described under Materials and Methods. The authenticity of the isolated fibroblast TF antigen was established by Western blot analysis. Filters were probed with monoclonal (Figure 1) and polyclonal (not shown) anti-TF antibodies. In both cases fibroblast TF appears slightly larger than brain TF. The difference was eliminated by first digesting the proteins with endoglycosidase F (results not shown); this result conforms to the suggestion that there is a single TF gene (Carson et al., 1985; Scarpati et al., 1987; Kao et al., 1988).

Fibroblast TF was subjected to SDS–PAGE and autoradiography (Figure 2). In accord with the Western blots, unreduced fibroblast TF is a single radioactive band migrating just above the tritiated human brain TF standard. Reduction with 2-mercaptoethanol liberated of most of tritium from fibroblast TF as expected for thioester-linked [^{3}H]palmitate (O'Brien et al., 1987).

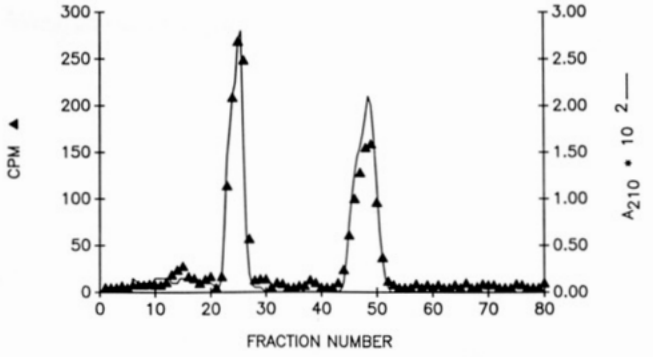

FIGURE 3: Reverse-phase HPLC analysis of the ^{3}H fatty acids covalently coupled to fibroblast TF. The ^{3}H fatty acid methyl esters derived from labeled fibroblast TF were resolved on a C_{18} reverse-phase column as described under Materials and Methods. The methyl palmitate and methyl stearate internal standards were detected by absorbance at 210 nm. The chromatographic profile of the ^{3}H fatty acid methyl esters was monitored by liquid scintillation counting. The peaks of absorbance and radioactivity eluting in fractions 22–27 and 44–51 correspond to methyl palmitate and methyl stearate, respectively. The trace of material that emerged before the methyl ester peaks has the retention time appropriate for the two unmodified fatty acids.

The identity of the radioactive material covalently bonded to fibroblast TF was determined by reverse-phase HPLC. The protein was first precipitated with trichloroacetic acid and then extracted with organic solvents to remove noncovalently bound tritium. Unlabeled palmitic and stearic acids were added as internal standards, and the sample was hydrolyzed in 6 N HCl. The hydrolysate was extracted with benzene, treated with methanolic HCl, and chromatographed (Figure 3). The methylated material contained 79% of the radioactivity present in the protein prior to hydrolysis. All the tritium was recovered from the reverse-phase column; 52% eluted as [^{3}H]methyl palmitate, 42% as [^{3}H]methyl stearate, and 6% as the unmodified ^{3}H fatty acids. Thus, fibroblast TF is acylated by both palmitic and stearic acids. The conversion of a portion of the labeled C_{16} fatty acid to the C_{18} derivative is not surprising since palmitoyl-CoA participates in both fatty acid chain elongation and protein transesterification (Berger & Schmidt, 1984; McIlhinney et al., 1985).

The heterodimeric form of human TF reported by other investigators is composed of the TF polypeptide disulfide-linked via Cys^{245} to another protein of 12–13 kDa (Morrissey et al., 1987; Carson, 1987; Carson et al., 1988). Although we have never seen this type of material in any of our preparations of human or bovine TF, traces of disulfide-bonded TF dimer are routinely observed (see Figures 1 and 2). Since intracellular disulfide linkages are highly improbable owing to the cytoplasmic reducing potential (Maher & Singer, 1986; Walters & Gilbert, 1986), TF dimers are probably generated by deacylation of Cys^{245} and interchain disulfide bond formation during purification. When fibroblast TF was rapidly isolated from freshly induced cell cultures, no dimer was observed. To determine if the disulfide-linked TF dimers are spontaneously generated by deacylation, TF was treated with hydroxylamine and allowed to oxidize. As shown in Figure 4, dimeric TF, a trace component of the starting material, is now the predominant species after hydroxylamine deacylation. The disappearance of this dimer following reduction or CNBr cleavage indicates that it is formed by an intermolecular disulfide bond involving Cys^{245}. This is consistent with a similar analysis of the heterodimer (Carson et al., 1988). The lack of spontaneous dimerization indicates that most of the Cys^{245} is, in fact, acylated, and generation of a free SH group by hydroxylamine indicates that the native protein was thioesterified.

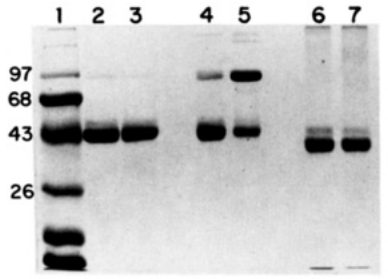

FIGURE 4: SDS–PAGE of hydroxylamine deacylated TF. Hydroxylamine deacylation, CNBr cleavage, and SDS–PAGE were performed as described under Materials and Methods. The lane assignments are as follows: (1) protein standards (see legend to Figure 1); (2 and 4) untreated TF; (3 and 5) deacylated TF; (6 and 7) CNBr digests of untreated and deacylated TF, respectively. Each TF lane contains 6 μg of protein. The samples in lanes 1–3 were reduced with 2-mercaptoethanol; lanes 4–7 were unreduced. The gel was stained with Coomassie Brilliant Blue R-250. Monomeric TF migrates at ≃45 kDa while the dimer is at ≃90 kDa. The minor band just above the TF monomer is not heterodimeric TF since it is still present after reduction. The band is apparently a minor glycosylated form of the TF monomer since it is recognized by the monoclonal antibody (Figure 1) and disappears following deglycosylation (not shown).

Two TF molecules must be in close proximity for the disulfide-linked dimer to be the favored product of Cys^{245} deacylation. The noncovalent complexation of TF was first suggested by direct binding studies where the interaction of TF and factor VIIa exhibited positive cooperativity (Bach et al., 1986; Fair & MacDonald, 1987). This self-association is directly confirmed by the homodimer described above (Figure 4). The genesis of the heterodimer may be viewed as a competing reaction. In one procedure (Carson et al., 1988) tissues were homogenized into buffers containing dithiothreitol, which promotes deacylation and disulfide exchange and is the probable cause of heterodimer formation. The details of the other purification (Morrissey et al., 1987) have not been published. In any event, our evidence indicates that the covalent dimers are most likely purification artifacts that are probably not present in vivo.

The palmitate or stearate on TF is covalently linked in the middle of a cluster of four positively charged amino acids abutting the hydrophobic domain of the protein. Anchoring these residues at the membrane interface may promote electrostatic interactions between the cytoplasmic tail of TF and lipid head groups, particularly since there are acidic phospholipids on the cytoplasmic side of the bilayer. Several other membrane proteins have similar acylated cytoplasmic domains. The modified half-cystines in the VSV G protein (Rose et al., 1984), the HLA-D-associated invariant chain (Koch & Hammerling, 1986), and the transferrin receptor (Jing & Trowbridge, 1987) are also near hydrophobic domains with neighboring positively charged amino acids. This structural motif may define at least one type of acylation site.

No role has been established for the acylation of integral membrane proteins. Since these proteins are already tightly associated with the lipid bilayer via hydrophobic amino acid sequences, the significance of an additional membrane anchor is not immediately obvious. The effect of deacylation on TF biological activity was examined by reconstituting the native and hydroxylamine-treated TF, which had dimerized, into phospholipid vesicles and measuring the specific procoagulant activity of each as described under Materials and Methods. The ratio of specific activities (untreated TF/deacylated TF) was 1.32 in PS/PC (30:70) vesicles and 1.31 in PC vesicles. Therefore, the procoagulant activity of dimerized TF was only slightly less than that of the single-chain form in vitro. Whether this posttranslational modification modulates any aspect of TF synthesis or expression in cultured cells or in vivo remains to be determined.

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Articles

Inactivation of Human Plasma Kallikrein and Factor XIa by Protein C Inhibitor†

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ABSTRACT: The inhibition of kallikrein and factor XIa by protein C inhibitor (PCI) was studied. The method of Suzuki et al. [Suzuki, K., Nishioka, J., & Hashimoto, S. (1983) *J. Biol. Chem. 258*, 163–168] for the purification of PCI was modified in order to avoid the generation of proteolytic activity and subsequent inactivation of PCI. With the use of soybean trypsin inhibitor, an efficient inhibitor of kallikrein and factor XIa, the generation of proteolytic activity was avoided. The kinetics for the inactivation of activated protein C (APC), kallikrein, and factor XIa by PCI were determined. In the absence of heparin, no inactivation of APC was observed, in contrast to kallikrein and factor XIa, which are inhibited with second-order rate constants of $(11 \pm 4) \times 10^4$ and $(0.94 \pm 0.07) \times 10^4$ M^{-1} s^{-1}, respectively. Addition of heparin potentiated the inhibition of APC [$(1.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1}] and factor XIa [$(9.1 \pm 0.7) \times 10^4$ M^{-1} s^{-1}] by PCI, whereas the inhibition of kallikrein by PCI was unchanged [$(10 \pm 1) \times 10^4$ M^{-1} s^{-1}]. The second-order rate constants for the inhibition of kallikrein or factor XIa by PCI were similar to the second-order rate constants for the inhibition of their isolated light chains by PCI, indicating a minor role for the heavy chains of both molecules in the inactivation reactions. With sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis and immunoblotting, complex formation of APC, kallikrein, and factor XIa with PCI could be demonstrated. APC and kallikrein formed 1:1 molar complexes with PCI. Factor XIa formed 1:1 and 1:2 molar complexes with PCI, indicating that both active sites on the factor XIa molecule can be inactivated by PCI. On the basis of second-order rate constant calculations, PCI would account for 7% of the inactivation of kallikrein and 5% of the inactivation of factor XIa in plasma, indicating that PCI can be physiologically important in the inhibition of both proteinases. Moreover, because of the high second-order rate constants for the inactivation of kallikrein and factor XIa by PCI and the low concentration of PCI in plasma, the PCI activity in plasma could be regulated by these proteinases of the contact system.

Activated protein C (APC)[1] is a vitamin K dependent serine protease that plays an important role in the regulation of blood coagulation (Stenflo, 1976; Kisiel et al., 1977; Kisiel, 1979; Dahlback & Stenflo, 1980; Vehar & Davie, 1980; Marlar et al., 1982, Suzuki et al., 1983b). In 1980, Marlar and Griffin described an inhibitor of activated protein C in plasma, the protein C inhibitor (PCI). PCI has been purified from human plasma by Suzuki et al. (1983a). It is a single-chain glycoprotein with a molecular weight of 57 000. PCI forms a 1:1 molar complex with APC. At the same time a small peptide at the carboxyl terminus is cleaved from PCI, resulting in a modified, inactive PCI (Suzuki et al., 1984). PCI inactivates not only APC but also thrombin and factor Xa. The action of protein C inhibitor is enhanced by heparin (Suzuki et al., 1984) and dextran sulfate (Suzuki, 1985). Recently, the cDNA for human protein C inhibitor has been characterized, and the amino acid sequence of PCI shows a high degree of homology with members of the superfamily of serine protease inhibitors, the serpins (Suzuki et al., 1987).

In this study we describe a purification procedure for PCI based on the method of Suzuki et al. (1983a). Plasma kallikrein and activated factor XI formed complexes with PCI. By use of specific inhibitors for these enzymes, inactivation of PCI during the purification was avoided. The kinetic

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[1] Abbreviations: εACA, ε-aminocaproic acid; APC, activated protein C; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; M_r, molecular weight; PAA, polyacrylamide; PCI, protein C inhibitor; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminoethane.

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