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THE FUNCTIONAL SITE OF PLACENTAL ANTICOAGULANT PROTEIN: ESSENTIAL HISTIDINE RESIDUE OF PLACENTAL ANTICOAGULANT PROTEIN [1]

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SUMMARY : Placental anticoagulant protein (PAP) rapidly lost its anticoagulant effect due to photooxidation in the presence of methylene blue at pH 7.9 and 8 °C. Photooxidized PAP failed to bind the phospholipid vesicle. It seemed unlikely that the protein underwent a change in molecular size during the photooxidation on the basis of its behavior in electrophoresis and gel filtration. Photooxidized PAP had significantly decreased histidine contents, whereas the contents of other amino acids remained essentially unchanged. The peptide, SHLRKV, was included in the functional site of PAP and still showed an anticoagulant activity. On the other hand, the peptide which substituted histidine by alanine, SALRKV, no longer showed the activity. It was shown that the histidine residue is involved in Ca^{2+} or the phospholipid binding site of the protein.

Placental anticoagulant protein (PAP), isolated from human placenta, inhibits the extrinsic and intrinsic pathways of blood coagulation and binds specifically to anionic phospholipid surfaces in a Ca^{2+}-dependent manner (1, 2). The binding of PAP to factor Xa, factor Va, and prothrombin was

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Abbreviations : PAP, placental anticoagulant protein ; PC, phosphatidylcholine ; PS, phosphatidylserine ; DFP, diisopropylfluorophoshate ; SBTI, soybean trypsin inhibitor ; PMSF, phenylmethanesulfonyl fluoride ; NEM, N-ethylmaleimide ; TLCK, N-tosyl-L-lysyl chloromethyl ketone ; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone ; PCMB, p-chloromercuribenzoic acid ; DTT, dithiothreitol ; IAA, monoiodoacetic acid ; EDTA, ethylenediaminetetraacetic acid.

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tested as described previously. The results excluded the possibility of strong or irreversible binding of PAP to these factors.

The same anticoagulant protein (calphobindin I) has been purified from human placenta and characterized by Iwasaki et al. (3), and similar proteins have been isolated from human liver (4) and umbilical cord arteries (5). This protein has also been purified from human placenta during studies of potential substrates of the epidermal growth factor receptor/kinase (termed endonexin II) (6, 7). Three additional anticoagulant proteins, PAP-II, PAP-III, and PAP-IV, were simultaneously isolated from human placenta (8). The nucleotide sequences of the human placental anticoagulant proteins PP4, VAC, and calphobindin II were reported by Grundmann et al. (9), Maurer-Fogy et al. (10), and Iwasaki et al. (11), respectively. Protein and cDNA sequence data (1, 3, 7, 9-12) show that PAP is a member of a described family of Ca^{2+}-dependent phospholipid-binding proteins variously termed lipocortins (13-15), calcimedines (16, 17), calpactins (18-20), protein I, II, and III (21, 22), calelectrins (23-25), annexins (26), p35 and p36 (27-29), or chromobindins (30). Recently, Pepinsky et al. reported that six distinct proteins have been characterized to date for these lipocortin families (15). Lipocortins differ from conventional Ca^{2+}-binding proteins in that they lack an EF-hand-type Ca^{2+}-binding site (31, 32), and they inhibit phospholipase A_2 activity and blood coagulation (33) .

The present paper describes the effect of chemical reagents, inhibitors, and photooxidation in the presence of methylene blue on PAP and the identification of the amino acids involved in the functional site of the protein. In the case of PAP, the functional site means the Ca^{2+} or phospholipid-binding site, because this protein does not show anticoagulant activity and phospholipid-binding ability in the absence of Ca^{2+}.

MATERIALS AND METHODS

Materials. PAP was purified from fresh human placenta as described previously (1). Materials were obtained from the following sources: Egg yolk phosphatidylcholine (PC) type V-E, bovine brain phosphatidylserine (PS), rabbit brain cephalin, aprotinin, diisopropylfluorophosphate (DFP), and soybean trypsin inhibitor (SBTI) were products of Sigma; trypsin was obtained from Miles Lab. LTD., Rep. of South Africa. NaB^3H_4 (319 mCi/mol) was purchased from New England Nuclear; methylene blue from Katayama Chemical Industries Co., Osaka; and phenylmethanesulfonyl fluoride (PMSF) from Eastman Kodak Co., Rochester, New York. N-ethylmaleimide (NEM) was purchased from Fluka AG, Buchs, Switzerland. N-tosyl-L-lysyl chloromethyl ketone (TLCK), N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), and p-chloromercuribenzoic acid (PCMB) were obtained from Wako

Pure Chemical Industries, Osaka. Antipain, chymostatin, pepstatin, elastatinal, amastatin, bestatin, and phosphoramidon were products of the Peptide Research Foundation, Osaka. Other reagents and organic solvents used were of analytical grade.

Clotting assay. Routine assays of anticoagulant activity of PAP were performed by the kaolin-activated partial thromboplastin time using rabbit brain cephalin as described previously (1).

Photooxidation. Photooxidation of PAP was performed according to the method of Takahashi et al. (34) as follows. To 0.5 ml of 0.01 % solution of PAP in 50 mM Tris-HCl, pH7.9, 0.5 ml of 0.1 % methylene blue was added, and the mixture was kept below 8 °C in an ice bath. The reaction mixture was irradiated from a distance of 15 cm with a 150 W incandescent lamp. Aliquots of 0.2 ml were withdrawn at appropriate time intervals for the assay of anticoagulant activity and amino acid analysis. Control experiments were performed under the same conditions without irradiation or methylene blue.

Amino acid analysis. The reaction mixture after photooxidation was lyophilized. The protein sample thus obtained was acid hydrolyzed with 6 N HCl gas for 2 h at 130 °C. After removal of the HCl, derivatization of the resultant amino acids with phenylisothiocyanate were carried out according to the method of Shoji et al. (35). The amino acids were determined with a Waters liquid chromatograph (Pico-Tag amino acid analyzer).

Phospholipid binding assay. Binding of PAP to phospholipid vesicles was measured by gel filtration as described previously (1).

Tryptic digestion of PAP. Enzymatic digestion of PAP was performed by incubating the protein at 37 °C in 50 mM Tris-HCl, pH7.9 for 24 hr with trypsin. The molar ratio between the protein and trypsin was 25 to 1. The resulting peptides were separated by HPLC using a Waters μBondasphere 5μ C18-100Å column (3.9 x 150 mm). A gradient system composed of 0.1 % trifluoroacetic acid (solvent A) and 0.1 % trifluoroacetic acid in 60 % acetonitorile (solvent B) was used for elution of the column at a flow rate of 0.5 mL/min. The effluents were monitored at 214 nm, and peptides were collected manually.

RESULTS AND DISCUSSION

Effect of chemical reagents on anticoagulant activity of PAP. PAP was allowed to react with a variety of chemical reagents as described previously. PAP showed anticoagulant activity in the presence of Ca^{2+}, and the activity was decreased with the addition of EDTA. PAP was not inhibited by DFP, PMSF or benzamidine, which are typical serine protease inhibitors. TLCK and TPCK, which are the specific modifiers of histidine residues located in the active sites of trypsin and chymotrypsin, respectively, did not affect the anticoagulant activity of PAP. PAP was not inhibited by DTT, IAA, NEM or PCMB, which are the inhibitors of SH-protease. Soybean trypsin inhibitor, pancreatic trypsin inhibitor

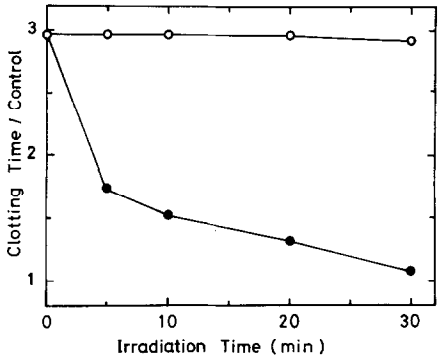

Figure 1. Time courses of photooxidative inactivation of PAP.
Placental anticoagulant protein (0.005%) in 50 mM Tris-HCl, pH7.9, was photooxidized in the presence of 0.05% methylene blue at 8 °C. The control experiment was performed under the same conditions without methylene blue. ●, with methylene blue; ○, without methylene blue.

(aprotinin) and the other peptidyl inhibitors like antipain, chymostatin, pepstatin, elastatinal, amastatin, bestatin and phosphoramidon did not inhibit the activity of PAP. 2-hydroxy-5-nitrobenzyl bromide (HNBB), a modifier of tryptophan residues, showed practically no effect on the activity of PAP after incubation for 6 or 24 hr.

Inactivation of anticoagulant activity of PAP by photooxidation. Figure 1 shows the time course of photooxidative inactivation of anticoagulant activity of PAP. PAP progressively lost its activity due to photooxidation in the presence of methylene blue. After 30 min of photooxidation, the activity of PAP had been lost almost completely, whereas no inactivation took place in the control experiments without methylene blue. Electrophoresis and gel filtration of PAP before and after photooxidation showed that neither significant change in molecular size nor cleavage of peptide bonds occurred durning photooxidation (data are not shown).

Binding of PAP to phospholipid vesicles. The binding capability of PAP to phospholipid before or after photooxidation was studied. Phospholipid vesicles were prepared by briefly sonicating a mixture of 20 % PS and 80 % PC as described previously (1). Binding experiments were performed with gel filtration on Sepharose 2B column in the presence of BSA and Ca^{2+} using [^{3}H] PAP before or after photooxidation. PAP bound specifically to phospholipid vesicles as reported previously (1). Before photooxidation, [^{3}H] PAP was detected in the void volume, demonstrating the binding of PAP to phospholipid vesicles (Figure 2A). However, photooxidized PAP no longer bound to phospholipid vesicles, and [^{3}H] PAP appeared in retarded fractions (Figure 2B). These experiments showed that the site of PAP binding to phospholipid was photooxidized in the presence of methylene blue, and the binding capability of PAP to phospholipid was lost.

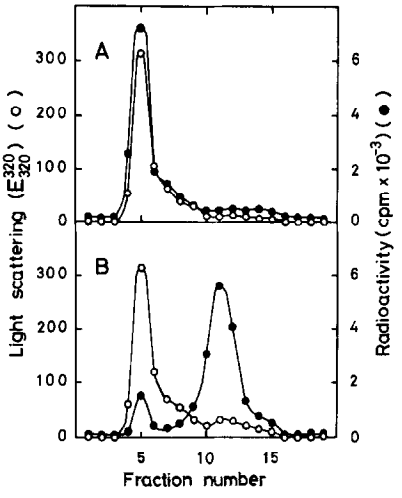

Figure 2. Binding of PAP to phospholipid vesicles.

The binding mixture contained 0.1 mg of phospholipid vesicles, 5 μg of [^{3}H] PAP (total 2.5 x 10^5 cpm), and 0.1 mg of BSA in 210 μL of Tris-HCl, pH7.9, containing 1 mM $CaCl_2$ and 50 mM NaCl. The binding mixtures were then applied to a column (0.7 x 14 cm) of Sepharose 2B. Elution was performed with 50 mM Tris-HCl, pH7.9, containing 50 mM NaCl at a flow rate of 10 mL/h. Fractions of 0.5 mL were collected. A, before photooxidation; B, after photooxidation.

Table 1. Amino Acid Composition of PAP Photooxidized in the Presence or Absence of Methylene Blue

Amino acid	No. of residues (moles per mole protein) a)								Theoretical values for native PAP
	Control (- methylene blue)				Photooxidized (+ methylene blue)				
	Irradiation time (min)				Irradiation time (min)				
	0	5	10	20	0	5	10	20	
Asp	29.5	28.7	29.4	30.0	28.8	29.5	31.4	30.3	31
Glu	39.6	39.3	38.7	39.6	39.3	39.0	41.4	39.8	41
Ser	19.9	20.4	19.7	21.4	20.9	21.2	21.8	21.0	21
Gly	24.1	26.7	26.3	24.1	22.5	23.2	24.1	22.2	22
His	3.3	3.5	3.7	3.4	3.4	NQ c)	NQ c)	NQ c)	3
Thr	21.7	20.4	23.2	21.9	21.3	23.1	22.0	22.4	23
Ala	27.6	27.1	27.6	28.1	29.4	31.2	31.4	30.1	26
Arg	17.9	18.2	20.0	18.7	18.2	18.8	19.6	18.2	19
Pro	6.1	6.0	5.9	5.6	5.2	5.8	5.1	5.7	5
Tyr	12.4	11.7	12.2	12.8	11.9	12.3	12.2	13.3	12
Val	14.7	15.6	15.4	15.2	15.6	16.7	17.3	17.4	16
Met	6.0	6.2	7.2	7.4	6.5	6.0	6.1	6.1	7
Cys(1/2)	0.8	1.0	1.1	0.7	1.2	1.1	1.2	1.0	1
Ile	18.4	17.6	20.2	18.2	17.8	17.0	17.7	17.8	18
Leu	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38.0	38
Phe	12.5	12.6	13.5	11.9	11.7	12.5	13.2	12.6	13
Lys	20.1	21.5	22.0	20.9	19.9	20.6	21.9	21.4	22
Trp b)				0.8				0.8	1
Total									319

a) The values are given in terms of molar ratio of amino acids, assuming the number of leucine residues to be 38.0. Acid hydrolysis was performed with 6 N HCl at 130°, 2 hr.

b) Determined by the ultraviolet spectrophotometric method.

c) Could not be quantitated.

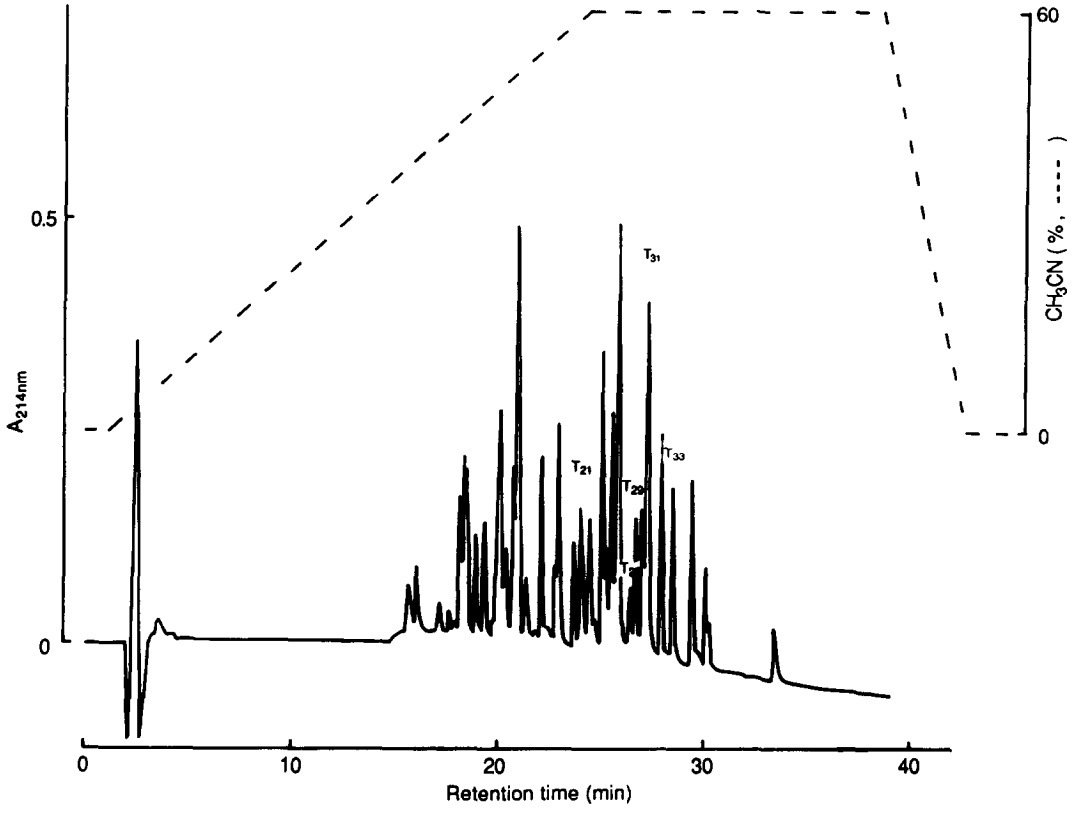

Figure 3. Tryptic peptide map of PAP.

A preparation of PAP containing 60 μg of protein was digested with trypsin. The cleavage products were separated by reverse-phase HPLC on a Bondasphere 5μ C_{18}-100 Å column (3.9 x 150 mm, Waters) as described under MATERIALS AND METHODS.

Amino acid composition of PAP photooxidized in the presence or absence of methylene blue. The results of amino acid analyses of acid hydrolysates of irradiated PAP in the presence or absence of methylene blue are shown in Table I. During the irradiation of PAP for 0-20 min at 8 °C and pH7.9, aliquots of the reaction mixtures were withdrawn at appropriate time intervals for amino acid analysis. The only significant changes were seen in histidine contents of PAP after irradiation in the presence of methylene blue. The contents of other amino acids were essentially unchanged. In control experiments, in the absence of methylene blue, all amino acids were unchanged. The results suggest that the histidine residue is essential for the anticoagulation activity and the binding to phospholipid of PAP.

Tryptic peptide map of PAP. Figure 3 shows a tryptic peptide map of PAP where cleaved products were separated by reverse-phase HPLC on a C_{18} column. All peaks were fractionated manually, and the anticoagulant activity for all fractions was assayed. The anticoagulant activity possessing

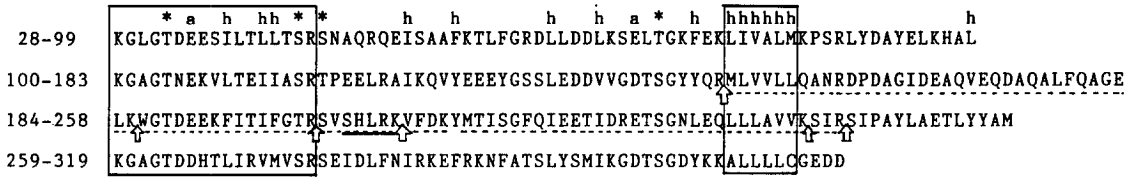

Figure 4. Alignment for the four repeated sequences in PAP.
The boxed regions correspond to the two consensus sequences of phospholipid binding proteins. The positions that have hydrophobic, hydroxy, and acidic amino acids are marked by h, asterisks, and a, respectively. Solid underline shows the minimum functional peptide that has an anticoagulant activity. Broken underline shows the functional peptide fragments obtained from PAP by tryptic digestion. ⇨, tryptic cleavage site.

peaks, T21, T28, T29, T31 and T33, were sequenced by amino acid analysis.

Alignment for the four repeated sequences in PAP. Figure 4 shows alignment for the four repeated sequences in PAP. Boxed regions in Figure 4 correspond to the two consensus sequences of phospholipid-binding proteins (12, 15). The first region, the 17 NH_2-terminal residues, is KGXGTDEXXhhXhhXSR, where h represents hydrophobic amino acids. This type of sequence has been found in the Ca^{2+}-dependent membrane-binding proteins, such as endonexin and calelectrin (26), and in lipocortins (13-15, 18, 19). The second homologous region in these proteins is a stretch of six residues of hydrophobic amino acids at the C-terminal portion of each repeat. These two regions are thought to be directly involved in binding to phospholipid (26). The results of amino acid

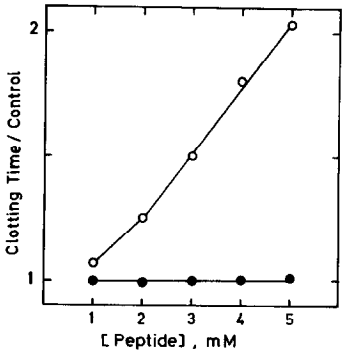

Figure 5. Effect of peptides on clotting activity.
The anticoagulant activities of the peptides were determined by kaolin-activated clotting times as described under MATERIALS AND METHODS. The peptide, SHLRKV, correspond from Ser-203 to Val-208 of the sequence of PAP; SALRKV, the peptide in which alanine is substituted for the histidine residue of SHLRKV. ○, SHLRKV; ●, SALRKV.

analysis of the active peptides show that T21 corresponds from Trp-186 to Lys-207; T28, from Ser-201 to Lys-241; T29, from Ser-201 to Arg-244; T31, from Trp-186 to Lys-241; and T33, from Arg-151 to Lys-207, of the sequence of PAP, respectively. The positions of the tryptic cleaved sites of the functional peptides are marked by arrows. The residues that are underlined included the functional peptides.

Effect of peptides on clotting activity. In order to ascertain the essential histidine residue, we synthesized a peptide, SHLRKV, which corresponded from Ser-203 to Val-208 of the sequence of PAP and assayed the effect of this peptide on kaolin-activated clotting times. Control experiments were performed using SALRKV, which substituted histidine by alanine. Figure 5 shows the results for the effect of these peptides on clotting activity. The peptide, SHLRKV, prolonged the kaolin-activated clotting time with increasing concentration. At a final concentration of 5 mM, the clotting time was prolonged from 95 to 202 seconds. This prolongation corresponded to a greater-than-85 % inhibition of factor Xa when it was calculated from a standard calibration curve constructed by serial dilution of factor Xa. On the other hand, SALRKV did not affect this clotting time.

These results show that the peptide, SHLRKV, is included in the functional site of PAP and exhibits an anticoagulant activity, but the peptide in which alanine is substituted for histidine does not. It seems that His-204 is an essential residue for anticoagulant activity of PAP. In addition to the two regions of each of the four repeating sequences of PAP, the histidine residue should be involved in Ca^{2+} or the phospholipid-binding site of the protein. This is the first report on the Ca^{2+} or phospholipid-binding residue of the family of Ca^{2+}-dependent phospholipid-binding proteins.

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