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ISOLATION AND PARTIAL CHARACTERIZATION OF A CYSTINE-RICH BASIC HEPARIN-BINDING PROTEIN FROM BOVINE PLATELETS

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SUMMARY We report the isolation and partial characterization of a so far unidentified basic platelet protein. Delipidated bovine platelets were extracted at pH 2.1. The extract was subjected to differential precipitation at pH 5.4–5.5 and by ammonium sulfate, then it was further purified by ion exchange chromatography on DEAE and CM cellulose columns in an urea containing medium. The major protein peak eluted from the CM cellulose column by NaCl gradient contained a protein in electrophoretically homogeneous form. It consists of a single polypeptide chain with an M_r of 28,000 as estimated by SDS PAGE. It was shown to be extremely rich in lysine and cystine and possessed a highly basic character (pI 9.8 – 10.1). On this basis the term cystine-rich basic protein (CRBP) was proposed for the new protein. Unlike some other low M_r basic proteins it did not bind calmodulin and troponin C, however, it showed significant heparin neutralizing activity.

So far, two proteins of highly basic character (isoelectric point around pH 10.0) – platelet derived growth factor (PDGF) and platelet basic protein (PBP) – have been isolated and relatively well characterized from platelets. PDGF is a heat stable, cationic (pI 9.5–10.4) glycoprotein with the molecular mass of approximately 30,000 daltons (1–7). It has growth promoting and chemotactic activity on vascular smooth muscle cells and fibroblasts (5,8,9,11,12), but it is not mitogenic for endothelial cells (5,10). PDGF is also chemotactic for monocytes and neutrophils (13). Reduction of disulfide bounds in PDGF produces biologically inactive, lower molecular size polipeptides in the range of 14,000–18,000 daltons (5,6,7). PDGF from human platelets is a heterodimer of A and B chains (14) while its porcine counterpart consists of B–B homodimers (15).

Using histone markers the M_r of PBP, the other highly cationic platelet protein (pI 10.0–10.5), was estimated to be 11,000–15,000 daltons, while comparing to conventional marker proteins it was found 14,000–17,000 (16). PBP binds heparin and it is the precursor of low affinity platelet factor 4 (LA–PF_4) and ß–tromboglobulin (16,17), two well–known heparin binding proteins.

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The abbreviations used are: AT III, antithrombin III; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) ; LA–PF4,low affinity platelet factor 4; PBP, platelet basic protein; PGDF, platelet derived growth factor; PF4, platelet factor 4; SDS PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEMED, N,N,N',N'–Tetramethylethylenediamine.

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In this paper we report the isolation and partial characterization of a third basic platelet protein for which, based on its amino acid composition, the terminology of cystine rich basic protein (CRBP) is proposed.

MATERIALS AND METHODS

Materials

Electrophoretically homogenous Antithrombin III (ATIII) was a kind gift of dr. Raymund Machovich. Aprotinin and highly purified bovine thrombin were the products of Boehringer Mannheim (Mannheim, Germany). M_r marker kit (Dalton Mark VI) containing bovine serum albumin (66 kD), ovalbumin (45 kD) pepsin (34.7 kD), trypsinogen (24 kD), ß–lactoglobulin (18.4 kD) and lysozyme (14.3 kD) was purchased from Sigma Chemical Co. (St. Louis, MO.). The pI Calibration kit "Electran" (pI range 4.7-10.6) was from BDH Chemicals Ltd. England. Troponin I and troponin C were isolated from rabbit skeletal muscle according to Perry et al (20). Calmodulin was purified from bovine brain (21). Three times washed platelets were obtained from freshly drawn bovine blood by differential centrifugation.

Electrophoretic techniques

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS PAGE) was performed according to Weber and Osborn (18) in 7.5% disc gel or by the method of Laemmli (18) in 10% slab gel. The calmodulin and TNC binding capacity of the platelet protein was examined by alkaline urea polyacrylamide gel electroforesis (22, 23) both in the presence and absence of Ca^{2+}.

Isoelectric focusing was carried out in 3.9% polyacrylamide gels by a slightly modified method of O'Farrell (24). Concentration of TEMED and ammonium persulfate was doubled. 0.2 M NaOH, 20 mM $Ca(OH)_2$ was used as cathode electrode solution, proteins were migrating toward the cathode (basic buffer in the bottom reservoir). The pH gradient was developed with ampholines pH 3.5-10, or pH 5.0-12.5 and the pI of isolated platelet protein was determined by using pI calibration protein standards in the range of pH 4.7-10.6.

Determination of amino acid composition

The isolated platelet protein was hydrolysed in 6 M HCl at 110°C for 24 h after oxidation with performic acid. Amino acid analysis was kindly performed by dr. László Patthy and his coworkers on a Biotronik LC 2000 analyser. The presence of tryptophan was demonstrated by the determination of fluorescence emission spectrum at 282 nm excitation wavelength using a Perkin-Elmer MPF-2L spectrofluorometer. Free cysteine content of the protein was determined with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in the presence of protein denaturing agents (6.7 M urea and/or 7.5 mg/ml SDS) (25, 26).

Assay of antiheparin activity

The heparin neutralizing activity of isolated platelet protein was determined in an ATIII-thrombin-heparin system using a chromogenic substrate (chromozym TH: Tos-Gly-Pro-Arg- p–nitroaniline) for the measurement of thrombin activity. The principle of heparin neutralizing assay is as follows: ATIII in the presence of heparin induces an immediate inhibition of equimolar thrombin. In the conditions used in the assay heparin is essential to this inhibition, i.e., without heparin no inhibitory activity of ATIII could be observed. The inhibiton of thrombin is relieved by the preincubation of heparin with heparin antagonist. As heparin was present in the assay system well below saturating concentration - it resulted only in 50% inhibition of thrombin activity - the extent of the decrease of inhibition quantitatively reflected heparin neutralization.

Various amonts of the isolated platelet protein in 25 µl 7 M urea (its pH was adjusted to 5.1 with HCl) or in 0.15 M NaCl adjusted to pH 2.1 with HCl was incubated for 1 min with 450 µl heparin (0.15 U/ml in a buffer containing 6.5 U/ml aprotinin, 10 mM EDTA, 150 mM NaCl, 100 mM Tris-HCl pH 8.1) and 25 µl ATIII (0.4 U/ml) at 25°C. Either purified ATIII preparation or human citrated plasma diluted to the above ATIII activity with physiological saline was used as the source of ATIII. To the mixture 25 µl thrombin (0.5 NIH U/ml) was added and after 2 min incubation residual thrombin activity was measured with a kinetic assay using chromozym TH chromogenic substrate (0.173 mM). The rate of p-nitroaniline release was measured at 405 nm. The results were expressed as percentage neutralized heparin and calculated according to the following formula:

$$HNA\% = \frac{\Delta A/min(A+H) - \Delta A/min\ (+H)}{\Delta A/min(-H)\ -\ \Delta A/min\ (+H)} \times 100$$

HNA: heparin neutralizing activity; (-H), (+H), (A+H): the assay was carried out with or without heparin or in the presence of heparin antagonist plus heparin, respectively.

RESULTS

Preparation of cystine-rich basic protein from bovine platelets

All procedures used prior to ion exchange chromatography were performed 0–4°C. 40 g washed bovine platelets were homogenized in 750 ml of chloroform-metanol (2:1, v/v) in a Waring blendor (2x1 minute) and the suspension was stirred overnight. The residue was collected by vacuum filtration, washed with 2x300 ml chloroform-metanol (2:1) and 2x250 ml acetone . The delipidated residue was extracted twice in 500 ml distilled water for 2x20 min, then centrifuged at 4,500 g for 20 minutes. The pellet was resuspended in 450 ml water, its pH was adjusted to 2.1 with 2M HCl (during lowering the pH, near pH 2.8, the solution became slightly gelatinous). After 3 hours stirring the solution was centrifuged at 30,000 g for 20 minutes. The supernatant was collected, the gelatinous residue was resuspended in 300 ml water (the pH was again adjusted to 2.1) and extrated for another 2 hours. The two pH 2.1 extracts were combined, and the pH was raised to 5.4-5.5 with 10 N NH_4OH. The solution was stirred for 1 hour and then centrifuged (5,000 g, 15 min) to remove the formed precipitate. The supernatant was fractionated with solid ammonium sulfate, added to 50% saturation (35.4 g/100 ml). After stirring for 4 hours the precipitate was collected by centrifugation (15,000 g, 15 min). It was washed with 50% saturated ammonium sulfate and finally taken up in 30-40 ml of water. Insoluble proteins were removed by centrifugation (15,000 g, 15 minutes), the supernatant was dialysed against dist. water to remove ammonia and lyophilized.

Further purification was carried out at room temperature by ion exchange chromatography. Lyophilized proteins were dissolved in 15-20 ml of 8 M urea, 15 mM ß-mercaptoethanol, 1 mM $CaCl_2$, 75 mM Tris-HCl, pH 8.0 buffer and the solution was applied to a DEAE-cellulose column (2.6x10 cm, Whatman DE32), equilibrated with the same buffer. At a flow rate of 50 ml/h 5 ml fractions were collected, and the absorbance was monitored at 280 nm. The protein fractions not bound to the DEAE cellulose column were pooled and the pH of the combined solution was adjusted to 5.1 with 1 M-citric acid. The solution was dialysed against 7 M urea, 1 mM $CaCl_2$, 7.5 mM ß-mercaptoethanol, 32.5 mM sodium citrate, 25 mM citric acid pH 5.1, and applied to a CM-cellulose column (2.6x10 cm; Whatman CM23) equilibrated with the above buffer. After the absorbance (at 280 nm) returned to the base-line the column was eluted with 2x150 ml NaCl gradient (0-0.4 M) in the same buffer (at 50 ml/l flow rate 5 ml fractions were collected). The elution profile is shown on Fig. 1. Fractions 11-22 were pooled and concentrated using Millipore CX 10 membrane, then dialysed against 3 mM HCl and freeze-dried. 1.0-1.5 mg electrophoretically homogenous protein (Fig.2) was eluted from the CM-cellulose column.

Partial characterization of isolated platelet protein

Mobility of the protein in SDS gel did not change significantly when the reducing agent was omitted from the sample buffer or when extremely high dithiothreitol or ß-mercaptoethanol

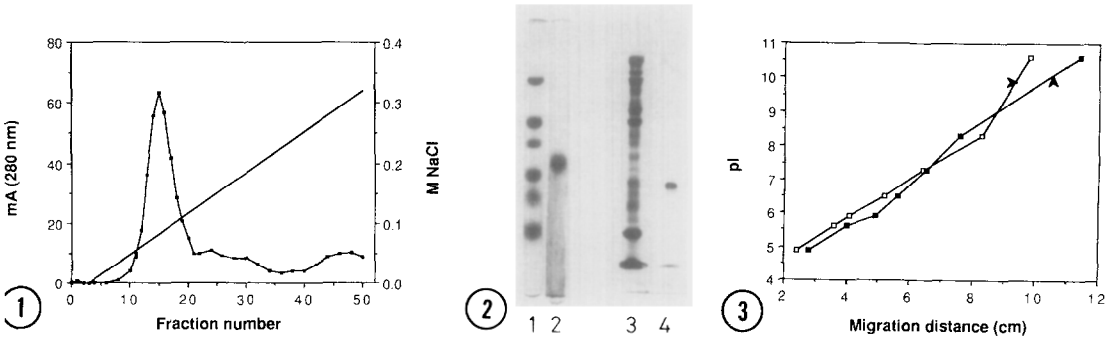

Fig. 1. The NaCl gradient elution profile of platelet proteins bound to CM–cellulose column. The optical density was measured at 280 nm. Fractions 11-22 were pooled and concentrated.

Fig. 2. SDS PAGE analysis of isolated platelet protein in 7.5% Weber-Osborn (lanes 1,2) and 10% Laemmli (lanes 3,4) gels. Lane 1: M_r markers: bovine serum albumin (66 kD), ovalbumin (45 kD), pepsin (34.7 kD), trypsinogen (24 kD), ß lactoglobulin (18.4 kD), lysozyme (14.3 kD); lane 2: 20 µg platelet protein isolated from the CM cellulose column; lane 3: protein preparation applied onto the CM cellulose column (100 µg); lane 4: 8 µg isolated platelet protein.

Fig. 3. The determination of isoelectric point of cystine–rich basic protein by isoelectric focusing using two different ampholine systems (❑, ■). The following PI marker proteins were used: C–phycocyanin (pI 4.75), azurin (pI 5.65), trifluoroacetylated met-myoglobin (porcine; pI 5.92), met-myoglobin (porcine; pI 6.45), met-myoglobin (equine; pI 7.3), met-myoglobin (sperm whale; pI 8.3), cytochrome C (horse heart; pI 10.6). Arrowheads mark the migration distance of CRBP in two different ampholine containing gels.

concentrations were used (not shown), which suggest that the protein consists of a single polipeptide chain. By SDS PAGE according to Weber and Osborn, in reducing condition, an M_r of 28 kD was established for the isolated platelet protein (calculation was based on comparison with conventional M_r markers; Fig. 2).

By isoelectric focusing in polyacrylamide gel, using two different ampholine systems the isolated protein was found of highly basic character with pI between 9.8-10.1 (Fig.3). This finding is supported by the result of the determination of amino acid composition (Table 1), being lysine the most common amino acid in the protein. The ratio of lysine to arginine was higher than 4. The protein contained an unexpectedly high number of half-cystine residue - the second most

Table 1. Amino acid composition of cystine–rich basic protein

Arginine	7	Alanine	20
Histidine	10	Glycine	20
Lysine	30	Proline	9
Phenylalanine	12	Glx[a]	24
Tyrosine	6	Threonine	14
Leucine	10	Serine	18
Isoleucine	6	Asx[b]	24
Valine	11	Half–cystine	26

The number of individual amino acid residues was calculated by assuming a molecular mass of 28,000 daltons.

a: glutamine plus glutamic acid.

b: asparagine plus aspartic acid.

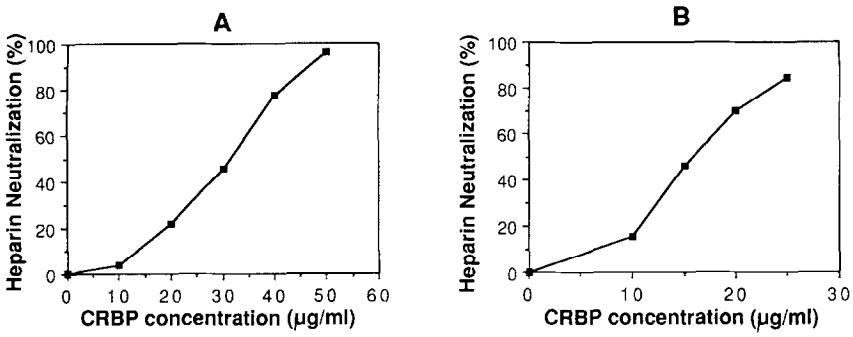

Fig. 4. Heparin neutralizing effect of cystine–rich basic protein in ATIII–thrombin–heparin system. The source of ATIII was either highly purified ATIII (A) or citrated human plasma (B). Results are expressed as percentage of heparin neutralized (see Materials and Methods for details).

common amino acid - but no free cysteine could be revealed by titration with DTNB in denaturing conditions, which suggests that all half-cystines form disulfide-bridges. Based on these findings we propose to term this protein as cystine–rich basic protein (CRBP). Following acid hydrolysis tryptophan could not be determined by amino acid analysis but the fluorescence emission spectrum of the protein was characteristic of tryptophan containing peptides (emission maximum at 350 nm). The amino acid analysis didn't differentiate between glutamine and glutamic acid, asparagine and aspartic acid but the highly basic character of CRBP suggests that these represent mainly glutamine and asparagine residues.

At physiological pH and ion concentration the solubility of highly purified CRBP is very poor, it can be kept in solution in concentrated urea solution or at low pH.

Functional studies

Looking for possible functions of CRBP we reasoned that as highly basic protein it might form complex with strongly acidic molecules of biological importance like calmodulin and/or heparin. The investigation of calmodulin (and troponin C) binding capability was carried out in alkaline urea polyacrylamide gels. No calmodulin or troponin C binding activity of CRBP could be demonstrated (not shown) while in the same conditions another basic protein, troponin I formed Ca^{2+} dependent complex with both calmodulin and troponin C. Results on the heparin neutralizing effect of CRBP are illustrated in Fig. 4. CRBP had a significant heparin neutralizing activity. It abolished the inhibition of thrombin caused by heparin-ATIII complex in a dose dependent manner. When only thrombin and ATIII was present in the system CRBP was without effect. Under the experimental conditions in a purified ATIII-thrombin-heparin system, 2.6 U heparin was neutralized by 1 mg CRBP. When plasma was used as the source of ATIII, i.e., when plasma proteins were also present, somewhat higher heparin neutralizing capacity (4.3 U/mg) could be detected. (Plasma proteins probably keep a higher portion of CRBP in solution under assay conditions which results in higher heparin binding capacity.)

DISCUSSION

We describe the purification and partial biochemical characterization of a highly cationic (pI 9.8-10.1) platelet protein, which was termed CRBP. The purified protein consists of a single

polypeptide chain (M_r 28 kD by SDS PAGE) and is distinct of other basic platelet proteins.

The identity of CRBP with PBP (pI 10.0-10.5) can be easily ruled out on the basis of their different molecular mass (11-15 kD for PBP).

By SDS PAGE in nonreducing conditions PDGF, the other strongly basic (pI 9.5-10.4) platelet protein has an M_r similar to that of CRBP, but it consists of two polipeptide chains held together by disulfide bridges and reduction results in two polypeptide chains with faster migration. Although, according to our knowledge, the amino acid composition of PDGF of bovine origin is not known, a comparison with the amino acid composition of human PDGF (2, 5) reveals such estential differences which further support that the two proteins are of distinct entity. In CRBP lysine is the predominant amino acid (ratio of lysine:arginine > 4), while the ratio of the two basic amino acids in PDGF is close to 1. The half-cystine and phenilalanine content of CRBP is much higher than that of PDGF while valine is present in PDGF in a much higher proportion than in CRBP. Moreover, in contrast to CRBP, PDGF lacks tryptophan and contains methionine. A further difference is that the heparin binding capacity of PDGF is neglible.

The heparin neutralizing activity of CRBP was investigated in an ATIII-thrombin-heparin system similar to the system designed by Rucinsky et al. (27). The latter authors found heparin binding capacities of 17 U/mg and 2.6 U/mg for platelet-factor 4 (PF4) and for low-affinity platelet factor (LA-PF4) respectively. The heparin binding capacity calculated on the basis of protein mass puts CRBP in the range of LA-PF4. When calculated on molar basis the heparin binding capacity of CRBP is between those of PF4 and LA-PF4, i.e. in in vitro conditions heparin binding by this protein is rather significant. At this stages it is not possible to establish if this property of CRBP is of any physiological or pathological significance. Experiments with specific antibodies raised against CRBP are to be carried out to establish the subcellular localization of this protein and to decide if it is released during platelet activation. A further question is if CRBP, like PDGF, have mitogenic property.

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