Studies on Platelet Proteins. IV. Some Physical and Chemical Properties of Thrombosthenin

By P. Ganguly

The importance of blood platelets in hemostasis is now well established.¹ Many of the physiologic properties of platelets suggest the presence of a contractile protein and Bettex-Galland and Lüscher (1961) succeeded in isolating such a contractile protein, thrombosthenin, and studied some of its properties.²–⁴ Grette (1962) confirmed the presence of this protein in pig platelets.⁵ Since then, a large number of papers have appeared reporting the ATPase activity,⁶ solubility, biosynthesis⁷ and immunologic properties⁸ of thrombosthenin and it is now generally considered that the microfibrils, responsible for maintaining platelet shape, consist of thrombosthenin.⁹–¹⁰ However, very little is known about the physicochemical properties of purified preparations of this important protein. This is largely due to its extreme lability and associated problems similar to those encountered with the contractile proteins from muscles. This communication reports studies on the isolation, stability and some physicochemical properties of thrombosthenin; in particular, sedimentation properties which have not to our knowledge been previously reported.

Materials and Methods

Thrombosthenin was isolated from human blood platelets following the method of Grette (1962)⁵. Whole human blood was collected in plastic bags as units of 500 ml. and contained 7 per cent ACDA. This was centrifuged at 2000 g. for 1 minute 40 seconds to separate the red cells. The platelet rich plasma was recentrifuged to isolate the platelets which were suspended in 15 ml. of plasma in plastic bags. Platelets from each bag were collected in separate tubes by centrifugation and washed three times with 1 per cent ammonium oxalate–0.1 per cent EDTA, pH 7.5 and once with 0.6 M KCl–0.02 M tris, pH 7.2. The mean recovery of platelets was 1 gm. (wet wt.) per unit of blood. To each gram of platelets 3 ml. of 0.6 M KCl–tris, pH 7.2 was added and the platelets were thoroughly suspended with a vortex mixer operated at low speed. To each ml. of the platelet suspension 25 µl. of butanol was added and the suspension was thoroughly mixed and left overnight at 10 C. After 20 hours, the suspension was centrifuged at 7710 g. for 5 minutes and the supernatant carefully removed. The sediment was resuspended in another 10 ml. KCl–tris and centrifuged at 12,100 g. for 15 minutes. The supernatant was removed and combined with the supernatant obtained earlier. Thrombosthenin was precipitated from this solution at 4 C. by adding two volumes of 0.002 M Mg²⁺ and adjusting the pH to 6.4 with dilute acetic acid. After 3 hours, the protein was collected by centrifugation and redissolved in 0.6 M KCl–tris. This step was repeated three times. The final product was further purified by filtration through a column (2.5 × 40 cm.) of Sepharose 4 B. The tubes containing the protein were pooled and concentrated by nega-
Fig. 1.—Sedimentation pattern of fresh thrombosthenin in 0.6 M KCl-tris, pH 7.2. Protein concentration 0.7 Gm./100 ml., 39,460 rpm., 30°. Patterns recorded at 10, 15, 20, 25 and 30 min. after start.

A fresh preparation of thrombosthenin in 0.6 M KCl-tris, pH 7.2 which had previously been precipitated three times showed multiple components in the ultracentrifuge. Three distinct hypersharp boundaries with sedimentation coefficients of 35 S, 56 S, and 83 S were noted at a thrombosthenin concentration of 0.7 Gm./100 ml. (Fig. 1). On gel filtration through Sepharose 4 B, this thrombosthenin sample showed a single symmetrical peak (Fig. 2). Based on six different experiments, using 0.6 M KCl-tris, pH 7.2 as the eluant, the average elution volume was 62 ± 3 ml. The eluted material at 0.3 Gm./100 ml. protein concentration showed a single boundary with a sedimentation coefficient of 36.5 S (Fig. 3). At higher protein concentration or with throm-
Fig. 2.—Typical gel filtration pattern of fresh thrombosthenin through Sepharose 4 B. Eluant 0.6 M KCl buffered with tris to pH 7.2. Fraction size 2.2 ml. The heavy bar on the abcissa shows the tubes pooled.

Fig. 3.—Sedimentation pattern of thrombosthenin after gel filtration in 0.6 M KCl-tris, pH 7.2. Same conditions as in Figure 1 except that the protein concentration was 0.3 Gm./100 ml. and the schlieren diaphragm angle 25°.

Thrombosthenin stored in 0.6 M KCl for more than 60 hours, multiple components were observed and the sedimentation pattern was similar to that presented in Figure 1. Contractile proteins of muscle are known to form aggregates at high protein concentrations. It is apparent that the 56 S and 83 S components are aggregation products of the basic unit with a sedimentation coefficient of 36 S.

With 1.0 M NaCl-tris, pH 7.2 as the eluant a single, symmetrical peak was again observed (Fig. 4). However, the elution volume under these conditions was 120 ± 4 ml. Thrombosthenin containing multiple components (see Fig. 1)
Fig. 4.—Gel filtration pattern of thrombosthenin stored 40 hrs. in 1.0 M NaCl at 10°C prior to application to a column of Sepharose 4 B. Same preparation as shown in Figure 1. Eluant 1.0 M NaCl-tris, pH 7.3. Fraction size 2.2 ml. The heavy bar on the abscissa indicates the tubes pooled.

Fig. 5.—Sedimentation pattern of thrombosthenin preparation shown in Figure 4. 56,100 rpm., 30°C, patterns recorded at 12, 15 and 20 min. after start.

when chromatographed using 1.0 M NaCl-tris as the eluant showed a single peak. Better resolution as judged by the shape of the peak, was obtained with 1.0 M NaCl than with 0.6 M KCl. In the ultra-centrifuge the material eluted with 1.0 M NaCl showed a single sharp boundary with a sedimentation coefficient of 18 S (Fig. 5). In three separate experiments with different preparations, the sedimentation coefficients obtained were 18 S, 17.1 S and 17.6 S.

When dialysed for 2 hours against distilled water in the cold the sedimentation properties of this material remained unchanged. Dialysis for longer periods led to aggregation and eventually precipitation of the protein. On
prolonged standing (78 hours) in 1.0 M NaCl at 10 C. the 18 S material further dissociated to a component with a sedimentation coefficient of 7 S (Fig. 6). This 7 S material showed all typical properties of thrombosthenin. The relative viscosity measured on four different preparations was 1.4 to 1.5 which dropped sharply on addition of 10^{-2} M ATP.

Thrombosthenin cannot be detected in the soluble part of fresh platelet homogenate in 0.2 M tris - HCl, pH 8.2. The presence of thrombosthenin in platelet membrane and granules has recently been reported. Attempts were made to detect thrombosthenin in the sediment after removal of the cell sap. For this purpose, the washed platelets were homogenized in a Potter-Elvehjem homogenizer and the homogenate centrifuged for 30 minutes at 30,000 rpm. The sediment was washed three times with tris buffer. This sediment after fractionation on a linear sucrose density gradient and subsequent examination of the bands under the electron microscope has been found to consist primarily of platelet membrane and granules. The greater part of the sediment was soluble in 0.2 M tris - HCl, pH 8.3 containing 0.2 per cent sodium dodecyl sulphate. To one ml. of this solution 50 units of bovine thrombin and to another ml. of the same solution an equal volume of buffer were added. After one hour at 4 C., the samples were analysed in the ultracentrifuge. The sedimentation pattern of such a preparation before and after thrombin treatment is shown in Figure 7. Three boundaries were observed with sedimentation coefficients of 3 S, 5 S and 8 S, the fastest of which was hypersharp in
Fig. 7.—Sedimentation pattern of platelet ghosts in 0.2 M tris-HCl, pH 8.3 containing 0.2 per cent sodium dodecyl sulphate. 59,780 rpm., 65, patterns recorded at 70, 75, 80 and 85 mins. The inversion of the fastest moving boundary may be due to lipid association.

![Sedimentation pattern of platelet ghosts](image)

Fig. 8.—Influence of ATP (10⁻² M) on the viscosity of thrombosthenin. Protein concentration 0.3 Gm./100 ml., temperature 20°.

nature and is, apparently, thrombosthenin. No significant difference in the thrombosthenin boundary was observed between the control and thrombin-treated samples. However, the second peak showed a distinct diminution in amount in the thrombin-treated sample indicating that it contained fibrinogen. These results differ from those reported by Bezkorovainy and Doherty on bovine platelet ghosts.¹⁴

A freshly prepared solution of thrombosthenin (0.3 Gm./100 ml.) had a relative viscosity of 1.77 which decreased sharply on addition of 2 per cent (v/v)
of $10^{-2}$ M ATP and returned to the original value after about 30 minutes. Addition of an equivalent amount of solvent did not produce any alteration in the viscosity. The specific viscosity of thrombosthenin was 0.7 to 0.8 and the ATP sensitivity varied between 50 to 80 per cent from preparation to preparation.  

In immunodiffusion against anti-thrombosthenin serum (a generous gift from Dr. R. L. Nachman, New York) adsorbed with human plasma and fibrinogen, purified thrombosthenin showed a single precipitation line close to the antigen well. However, the line was very faint presumably due to the high salt concentration necessary to prevent precipitation of thrombosthenin. No precipitin line was observed with purified thrombosthenin and anti-whole serum or antifibrinogen serum.

Purified thrombosthenin did not show a typical protein spectrum with an optimum at 280 m,$\mu$. The optical density at 260 m,$\mu$ was considerable producing a plateau in the 260–280 m,$\mu$ range. This may be due to materials with high absorption at 260 m,$\mu$ bound to the protein.

**DISCUSSION**

It is evident from studies in recent years that both the intracellular and membrane proteins play important roles in the physiologic function of blood platelets. Thrombosthenin has been suggested as being responsible for maintaining the platelet shape which is vital for physiologic function.$^{10,15}$ Anti-serum to thrombosthenin has been reported to inhibit clot retraction indicating that this protein is an important component of the clot retraction system.$^8$

Physical and chemical studies of thrombosthenin have proved to be difficult primarily due to its extreme instability and easy formation of aggregates. The formation of these multimers further reduces the stability of the protein. The refrigerator life of thrombosthenin in 0.6 M KCl, pH 7.2 is about 4 days after which it begins to precipitate out in the form of a white gel which is difficult to redissolve. Moreover, thrombosthenin isolated by either of the published procedures is often contaminated with fibrinogen. Gel filtration of such thrombosthenin preparations using 1.0 M NaCl as eluant, effects better resolution than 0.6 M KCl. We have been able to preserve thrombosthenin in 1.0 M NaCl-tris, pH 7.2 for more than ten days without any alteration in its properties. 1.0 M NaCl has the added advantage that it prevents formation of aggregates and breaks down the existing aggregates into a basic unit.

The sharpness of the sedimenting boundary obtained in the present study indicates thrombosthenin to be a highly asymmetric molecule which readily forms aggregates at high protein concentration. An unpublished report has also noted the presence of poorly defined complexes of varying sizes.$^{16}$ That these heavier components are actually aggregates of the same basic unit is evident from Figure 1 where the same preparation at low protein concentration showed a single boundary of 36 S. The 36 S boundary was always observed with extractions made in 0.6 M KCl. Electron microscopy of partially purified thrombosthenin has shown the presence of fibrilar structures (80 to 100 Å wide) similar to the microfibrils found in the cytoplasm and pseudopods of intact platelets.$^{17}$ It thus appears that thrombosthenin in vivo exists
as a large molecule of repeating units with 36 S unit as the most commonly characterized form. However at high ionic strength the 36 S unit again breaks down first to 18 S and then to 7 S units. Substantial ATPase activity comparable to thrombosthenin, was retained at the 7 S level whereas none of the thrombosthenin components (A and M) show ATP sensitivity. The presence of a kinetic unit with similar sedimentation characteristics in platelet ghosts solubilized in the presence of detergents (Fig. 7) suggests that the 7 S unit is the actual thrombosthenin molecule but that this easily aggregates to form higher components.

Since the specific viscosity, $\eta_{sp}$, of thrombosthenin reported in this communication is different from the literature values, a note of explanation appears to be worthwhile. The relative viscosity has always been in the range 1.7 to 1.9, although the values of the specific viscosity reported earlier are 0.1 to 0.2 (concentration expressed in g./1). If the relative viscosity of thrombosthenin (0.3 per cent) is 1.8, then the specific viscosity is, by definition, 0.8. The intrinsic viscosity [$\eta$] can be obtained from the slope of the curve of $\eta_{rel}$ against $c$ or from the intercept by plotting $\eta_{sp}/c$ against $c$. An equivalent result is obtained by extrapolating $1/c \ln \eta_{rel}$ to zero concentration, for in the limit approaching zero, $\ln \eta_{rel}$ is equal to $\eta_{sp}$. This standard nomenclature has been used for obtaining [$\eta$] in this study.

Direct estimation of molecular weight of thrombosthenin by Archibald or sedimentation equilibrium methods was not possible due to the high salt concentration, low protein concentration and the possibility of the presence of aggregates which would lead to an artificially high value for the molecular weight. The only information on the molecular weight of thrombosthenin available from previous studies is that the active material is excluded from P-300 gel indicating that its molecular weight is higher than 300,000 (ref. 8). An estimate of the molecular weight can be made by combining the viscosity and sedimentation data. Due to the presence of aggregates the main source of error in such a calculation will be in the value of the intrinsic viscosity[$\eta$]. To reduce this error, the protein sample was centrifuged for 30 minutes at 40,000 rpm. and only the top half of the supernatant collected. Two separate sets of measurement on two such samples were made and the mean intrinsic viscosity was determined to be 1.3 dl/g. With this value of [$\eta$], $\eta_{20, w} = 7.5, \beta = 2.2$ and $\nu = 0.728$ (ref. 22) the molecular weight of thrombosthenin is $8.9 \times 10^6$. This value of M should at present be considered approximate. An accurate determination of M must await precise determination of the parameters involved and complete purification of the protein when direct measurements can be made.

**SUMMARY**

The contractile platelet protein thrombosthenin has been isolated with butanol and purified further by repeated precipitation and gel filtration. Thrombosthenin thus isolated shows all the typical properties of this protein as reported in the literature. 1 M NaCl-tris, pH 7.2 has been found to be a better solvent than 0.6 M KCl as far as stability, resolution and aggregation of the protein are concerned. In the ultracentrifuge, 0.7 per cent thrombosthenin
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in 0.6 M KCl shows three hypersharp boundaries with sedimentation coefficients of 36 S, 56 S and 83 S. However, at low protein concentration only the 36 S boundary is observed. In 1 M NaCl, all these components break down producing a single kinetic unit of 18 S which dissociates further to 7 S. The 7 S component retains the ATP-sensitivity typical of thrombosthenin, suggesting it to be the monomeric unit of this protein. A similar hypersharp boundary with similar sedimentation coefficient to purified thrombosthenin has been noted in platelet ghosts solubilized in presence of detergent. A combination of sedimentation and viscosity data leads to an approximate molecular weight 8.9 × 10^5 for thrombosthenin.

SUMMARIO IN INTERLINGUA

Le contractil proteina plachettal thrombosthenina esseva isolate con butanol e purificata additionalmente per precipitation repetite e filtration a gel. Thrombosthenina assi isolate monstra omne le proprietates typic de iste proteina como illos ha essite reportate in le litteratura. Esseva trovate che 1 M NaCl-tris, a pH 7.2, es un melior solvente que 0.6 M KCl quanto a stabilitate, resolution, e aggregation del proteina. In ultracentrifugation, 0.7 procento thrombosthenina in 0.6 M KCl monstra tres hyperacute limines con coefficientes de sedimentation de 36 S, 56 S, e 83 S. Tamen, a base concentrationes de proteina, solo le limine a 36 S es observate. In 1 M NaCl, omne iste componentes decade e produce un sol unitate kinetic de 18 S le qual se dissocia additionalmente a 7 S. Le componente 7 S retine le sensibilitate pro triphosphato de adenosina typic de thrombosthenin, lo que suggestiona que illo es le unitate monomeric de iste proteina. Un simile limine hyperacute con un simile coefficiente de sedimentation a thrombosthenia purificate eseva notate in phantomas plachettal solubilisate in le presentia de detergent. Un combination de datos de sedimentation e de viscositate duce a un approximative peso molecular de 8.9 x 10^5 pro thrombosthenina.

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