Characterization of a Platelet Protein Phosphorylated during the Thrombin-Induced Release Reaction†

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ABSTRACT: Thrombin treatment of $^{32}PO_4^{3-}$-loaded, washed human platelets results in a two-to sixfold increase in phosphorylation of two proteins of approximately 40000 and 20000 M_r (designated "peak 7 protein" and "peak 9 protein", or P7P and P9P, respectively) as assessed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (Lyons, R. M., Stanford, N., & Majerus, P. W. (1975) *J. Clin. Invest. 56*, 924–936). We have purified P7P by preparative $NaDodSO_4$ gel electrophoresis sequentially in two different systems. The resulting homogeneous protein, purified 136-fold with yield of 3.4%, comprised 0.65% of the total platelet protein. P7P contained 0.3–0.5 mol of phosphate/mol of protein and had a major isoelectric point of 6.82. Despite the neutral isoelectric point, P7P contained 23.8% acidic residues and 14% basic residues, suggesting that there is likely considerable amidation of aspartate and glutamate residues. We compared the characteristics of P7P with those of several platelet proteins with some features in common with P7P. P7P differed significantly from actin in 10 of 16 amino acids and did not contain any 3-methylhistidine residues, an amino acid characteristically present in actin. These data conclusively indicate that P7P is not platelet actin. P7P and tubulin did not comigrate in $NaDodSO_4$ gel electrophoresis and the published amino acid analysis of tubulin differed from P7P by 20% in 8 of 16 amino acid residues. cAMP-binding proteins in platelet soluble protein did not coelectrophorese with P7P indicating that P7P is not the type II regulatory subunit of a cAMP-dependent protein kinase. Thus, the identity and function of this major soluble platelet phoshoprotein and its possible role in the platelet release reaction remain unknown.

Platelets can be stimulated to secrete specific substances without cell lysis (release reaction) and to aggregate into a mass of cells to form the hemostatic plug (Grette, 1962). We have suggested that phosphorylation of specific platelet proteins may play an important role in control of the platelet release reaction (Lyons & Atherton, 1978; Lyons & Majerus, 1974; Lyons et al., 1975). We reported that approximately 20 bands of radioactivity were detected when intact human platelets were loaded with $^{32}PO_4^{3-}$ and then fractionated by $NaDodSO_4$ gel electrophoresis[1] (Lyons et al., 1975). Radioactivity in two proteins, of M_r approximately 40000 and 20000 (termed peak 7 protein and peak 9 protein or P7P and P9P), was consistently increased (two- to sixfold) by exposure of the intact platelets to agents which induce the release reaction. Inconsistent increases in radioactivity in other bands were observed, but these were rarely more than 1.7-fold. Phosphorylation of P7P and P9P occurred with a time course and at a thrombin concentration similar to thrombin-induced serotonin release (release I) (Lyons et al., 1975; Daniel et al., 1977). Others confirmed these observations and showed that phosphorylation preceded aggregation and release of acid hydrolases (release II) (Daniel et al., 1977; Haslam & Lynham, 1976; Haslam & Lynham, 1977; Chiang et al., 1977). Phosphorylation occurred normally when release was induced by collagen in the absence of aggregation (Haslam & Lynham, 1977), while no phosphorylation was seen when aggregation was induced by ADP in the absence of release (Haslam & Lynham, 1976). These studies suggest that phosphorylation of P7P and P9P may be an important step in the control of the platelet release reaction but is not necessary for ADP-induced aggregation.

P9P has been identified as a light chain of platelet myosin (Daniel et al., 1977). When myosin light chain is phosphorylated, myosin demonstrates an increased actin-activated ATPase activity which may be responsible for the contractile phenomena associated with the platelet release reaction (Conti & Adelstein, 1975).

We report here that P7P can be purified to homogeneity by preparative $NaDodSO_4$ gel electrophoresis after preliminary purification steps in a buffer designed to stabilize P7P over a prolonged time period. Preparative $NaDodSO_4$ gel electrophoresis is carried out sequentially in two systems in which P7P migrates at different apparent M_r. The homogeneous protein contains 0.3–0.5 mol of phosphate/mol of protein and has a neutral isoelectric point despite an excess of acidic amino acid residues. These latter data suggest that there is considerable amidation of aspartate and glutamate residues.

Further, we have compared the physicochemical properties of P7P to three platelet proteins in an attempt to identify P7P. Our data suggest that P7P is neither the β subunit of tubulin nor the regulatory subunit of a cAMP-dependent protein kinase. Additionally, our data conclusively indicate that P7P is not a phosphorylated form of platelet actin.

Methods

Isotopes were purchased from the following sources: [γ-^{32}P]ATP (5 Ci/mmol), serotonin [2-^{14}C]binoxalate (27.5 mCi/mmol), [^{3}H]cAMP (20 Ci/mmol), and carrier-free $H_3{}^{32}PO_4$ (50 mCi/mL), New England Nuclear, Boston, MA; carrier-free sodium [^{125}I]iodide (50 mCi/mL), Mallinckrodt Chemical Works, St. Louis, MO; 8-azidoadenosine 3′,5′-monophosphate [^{32}P]triethylammonium salt (70.4 Ci/mmol), International Chemical and Nuclear, Irvine, CA. Kodak XR-5 X-Omat R medical X-ray film was obtained from Eastman Kodak Co., Rochester, NY. AG 501-X8 mixed bed ion-exchange resin was purchased from Bio-Rad Laboratories, Richmond, CA. Millipore filters (RAWP-025-000, 1.2-μm

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[1] Abbreviations used: $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; bisacrylamide, *N,N′*-methylenebisacrylamide; % T, total acrylamide; % C, percentage of bisacrylamide in the total acrylamide; DFP, diisopropyl fluorophosphate; PAS, periodic acid–Schiff; cAMP, adenosine 3′:5′-monophosphate; 8-N_3-cAMP, 8-azidoadenosine 3′:5′-monophosphate.

pore size, 25-mm diameter) were purchased from Millipore Corp., Bedford, MA. $NaDodSO_4$ (Sequanal grade) was purchased from Pierce Chemical Co., Rockford, IL. DNase I from bovine pancreas was purchased from Sigma Chemical, St. Louis, MO, and fluorescamine (Fluram) was purchased from Roche Diagnostics, Nutley, NJ.

Homogeneous human thrombin with a clotting activity of 2600 U/mg was a gift from Dr. John Fenton. Tos-$PheCH_2Cl$-trypsin was purified by the method of Kostka & Carpenter (1964). Homogeneous bovine renal cortical tubulin was a gift from Dr. Larry Barnes.

Platelet Preparation and $^{32}PO_4^{3-}$ Loading. Platelets were isolated by differential centrifugation by a modification of our previously described method starting with fresh, EDTA-anticoagulated human blood or with 3–5 day old citrate-anticoagulated human platelet concentrates (Lyons et al., 1975). The isolation was carried out at room temperature in isotonic, citrate-buffered saline, pH 6.5, containing 6.85 mM citric acid, 1.94 mM NaOH, 130 mM NaCl, 4 mM KCl, and 5.5 mM glucose. Large scale preparations were carried out in 450-mL polypropylene bottles in the JA10 rotor of a Beckman J21B centrifuge. The isolated platelets were resuspended in isotonic Tris-buffered saline, pH 7.4, containing 140 mM NaCl, 15 mM Tris-HCl, and 5.5 mM glucose ("resuspension buffer"), and the platelet concentration was estimated from the packed cell volume (Baenziger & Majerus, 1974). The platelet suspension was diluted to $2–6 \times 10^9$ platelets/mL and adjusted to contain 0.01–0.2 mCi/mL of $H_3{}^{32}PO_4$ at pH 7–7.5. Incubation at room temperature for 60 min served to load the platelets with $^{32}PO_4^{3-}$. Experiments requiring small volumes of platelets were carried out in a total volume of 0.025–0.125 mL in either 500-μL polyethylene or 1.5-mL polypropylene microtest tubes (Brinkmann Instruments, Inc., Westbury, NY).

Analytical $NaDodSO_4$ Gel Electrophoresis. Acrylamide (Eastman-5521) was recrystalized from chloroform by the method of Loening (1967). A stock solution containing recrystalized acrylamide and bisacrylamide (Sigma, St. Louis, MO) at the appropriate concentration was passed over a column of mixed bed ion-exchange resin and then stored at 4 °C in a brown bottle.

Platelets or platelet proteins to be fractionated by $NaDodSO_4$ gel electrophoresis were solubilized by boiling for 5 min in $NaDodSO_4$ buffer, i.e., 2% $NaDodSO_4$, 0.1 M 2-mercaptoethanol, 5.6% sucrose, 0.003% bromphenol blue, and one-fifth the concentration of buffer used in the well-forming gel in any of the systems described below. Samples that were not electrophoresed immediately were stored at −20 °C and heated again for 1 min before use.

$NaDodSO_4$ gel electrophoresis using the Weber–Osborn system (0.1 M sodium phosphate, pH 7.4; Weber & Osborn, 1969) was performed in either 10-cm long, 6-mm inside diameter glass tubes or in a 1.2-mm thick slab gel with 15 sample wells. The gels were stained overnight in 12% trichloroacetic acid, 50% methanol, and 0.03% Coomassie Brilliant Blue R 250 and subsequently destained by diffusion over 24 h in 7% acetic acid, 10% methanol. The cylindrical gels were frozen and divided into 2-mm slices with a razor blade slicer (Bio-Rad Laboratories), and the slices were assayed for [^{32}P]phosphate in a liquid scintillation counter after the addition of Bray's solution (Bray, 1960). $NaDodSO_4$–polyacrylamide slab gel electrophoresis was performed by a modification (Lyons et al., 1975) of the method of Weber & Osborn (1969) in an apparatus similar to that described by Reid & Bieleski (1968) purchased from Aquebogue Machine Works, Long Island, NY. The well-forming gel consisted of 4.5% T with 2.6% C (Hjertén et al., 1969) and 0.03% $NaDodSO_4$ in 0.05 M sodium phosphate buffer, pH 6.4. This acrylamide concentration was used in all of the stacking-well-forming gels described below. Staining and destaining were performed as for cylindrical gels. The gels were dried on Whatman no. 50 hardened filter paper with a Savant gel drier (Savant, Hicksville, NY) and exposed to Kodak XR-5 X-Omat R medical X-ray film that was subsequently developed with a Kodak RPX-Omat processor.

$NaDodSO_4$ gel electrophoresis was also carried out in the continuous neutral pH system of Fairbanks (Fairbanks et al., 1971) and the discontinuous alkaline pH systems described by Laemmli (1970) and Neville (1971). $NaDodSO_4$ was not included in the gel. Electrophoresis was carried out at 50 V until the sample had entered the stacking-well-forming gel and 100 V thereafter.

M_r were determined by $NaDodSO_4$ gel electrophoresis by plotting log M_r against R_f assuming a sigmoid curve (Rodbard, 1976). The best fit for the lines were calculated by the least-squares method (Snedecor & Cochran, 1967) using a computer program. Standard proteins for determination of M_r included (1) fibrinogen α chain, 73 000; (2) bovine serum albumin, 68 000; (3) fibrinogen β chain, 60 000; (4) fibrinogen γ chain, 53 000; (5) IgG H chain, 52 000; (6) human platelet actin, 42 000; (7) ovalbumin, 43 000; (8) aldolase, 40 000; (9) IgG L chain, 22 500; and (10) sperm whale myoglobin, 17 200.

Preparative $NaDodSO_4$ Gel Electrophoresis. Preparative gel electrophoresis in the Buchler Polyprep 200 apparatus (Buchler Inst., Fort Lee, NJ) was carried out as described by Chrambach (Chrambach et al., 1976). The Neville gel system was used with a 10-cm high, 11% T, 0.9% C separating gel, and a 2-cm high stacking gel. The gel was eluted at a flow rate of 1 mL/min with the lower bath buffer supplemented with 20% sucrose and 0.03% $NaDodSO_4$.

Preparative $NaDodSO_4$ gel electrophoresis in slab gels was carried out as described for analytical gels with the exception that only a single well was present. In early studies, the location of P7P in the preparative slab gel was detected by the presence of a Coomassie blue staining band at the expected R_f. Because of the low recovery at this step, P7P was located in later purifications by fluorescence using a modification of the method of Danno (1977). Two percent of the total protein at approximately 0.5 mg/mL in 5 mM sodium phosphate buffer, pH 7.4, 1.0% $NaDodSO_4$ was rapidly mixed with half its volume of fluorescamine, 1.5 mg/mL, in dioxane. The fluorescamine-labeled protein was then mixed with the unlabeled protein before stacking buffer and 0.1 M 2-mercaptoethanol were added. It was important to limit the amount of fluorescamine labeled protein to 2% because this level of labeling does not significantly alter either measurement of the isoelectric point (Danno, 1977) or the amino acid analysis (Mendez & Lai, 1975). P7P was carefully cut from the slab gel with a tissue slicer blade under an ultraviolet lamp with a wavelength of 360 nm and then eluted electrophoretically (Kårsnäs & Roos, 1977). The protein was dialyzed against 100 volumes of 95% ethanol to remove $NaDodSO_4$ and then four times against 100 volumes of 0.1 M ammonium bicarbonate buffer, pH 7.6, to remove nonvolatile salts and bromphenol blue. The protein was concentrated by lyophylization and redissolved by heating at 100 °C for 1 min in a small volume of 0.1% $NaDodSO_4$ in water. Recovery was generally about 85% when the fluorescamine localization method was used.

Methods of Platelet Disruption. Platelets were disrupted by two different techniques. Platelet suspensions were sonicated for 15 s on ice using the microprobe of a Biosonik IV

sonifier set at 70% intensity for volumes less than 3 mL and the standard probe at 100% intensity for volumes greater than 3 mL (Baenziger & Majerus, 1974). Alternatively, platelet suspensions were freeze-thawed three times.

Cellular Fractionation. Platelets were loaded with $^{32}PO_4^{3-}$ and treated with thrombin before disruption at 4 °C. During the disruption process, the samples were adjusted to contain inhibitor buffer (see legend to Figure 1) and 10 mM DFP. The DFP was added undiluted since the usual diluent, anhydrous isopropyl alcohol, altered the distribution of some platelet proteins.

Platelets were separated into particulate and soluble fractions by centrifugation at 200000*g* for 60 min at 4 °C and the particulate material was resuspended in the same volume and type of buffer as the soluble material. Both fractions were boiled in $NaDodSO_4$ buffer and aliquots electrophoresed on $NaDodSO_4$–polyacrylamide gels.

Trypsin Treatment of Intact Platelets. Intact platelets were treated with trypsin to hydrolyze susceptible proteins from the platelet surface. Reaction mixtures containing 0.1 mL of $^{32}PO_4^{3-}$-loaded platelets were exposed to thrombin, 1 U/mL, for 3 min followed by Tos-PheCH_2Cl-trypsin at a final concentration of 0.05–1.0 mg/mL. After 30 min at 37 °C, lima bean trypsin inhibitor (Sigma, type IIL) was added on a mg for mg basis of inhibitor to enzyme and the platelets were sedimented at 2250*g* for 5 min at room temperature. The supernate was removed and the pellet was suspended in resuspension buffer to the same volume as the supernate and both samples were adjusted to contain 10 mM DFP. The samples were then heated in $NaDodSO_4$ buffer in preparation for $NaDodSO_4$ gel electrophoresis.

Labeling of Intact Platelets with [γ-^{32}P]ATP. In an attempt to phosphorylate the platelet surface, 0.025-mL aliquots of platelets at 2×10^9/mL were incubated for 10 min at 22 °C with 0.1 mM [γ-^{32}P]ATP (600 mCi/mol) in resuspension buffer containing 4.5 mM magnesium acetate. The reaction mixture was adjusted to contain 4.5 μM cAMP, or 1600 U/mL of rabbit muscle protein kinase, or 1600 U/mL of rabbit muscle protein kinase and 4.3 μM cAMP. In some experiments, the platelets were pretreated with 1 U/mL of thrombin for 3 min. The reaction was stopped by boiling in $NaDodSO_4$ buffer.

Amino Acid Analysis. P7P and actin were hydrolyzed in vacuo at 110 °C for 24 h in 1 mL of 6 N HCl including 0.2% (v/v) phenol. The hydrolysate was analyzed on a Durrum D500 amino acid analyzer (Durrum Instruments Corp., Sunnyvale, CA). 3-Methylhistidine eluted from the column immediately after histidine. Serine and threonine were corrected for estimated losses of 10 and 5%, respectively. Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Norleucine was included in each sample before hydrolysis to provide an internal standard for losses during preparation of the hydrolysate.

Sucrose Density Gradient Centrifugation. $^{32}PO_4^{3-}$-loaded, thrombin-treated platelets were sonicated in inhibitor buffer and the soluble fraction obtained by centrifugation at 200000*g* for 60 min at 4 °C. Three hundred microliters of platelet soluble protein from 0.6×10^9 platelets (containing 150 μg of each of the following: rabbit muscle aldolase, M_r 160 000; bovine serum albumin, M_r 68 000; and ovalbumin, M_r 43 000) was layered onto a 12-mL, 5–20% sucrose gradient in inhibitor buffer and centrifuged at 285000*g* for 16 h at 4 °C. Fractions were collected from the bottom of the tube and a 30-μL aliquot from each 8-drop fraction was analyzed by $NaDodSO_4$ gel electrophoresis. The M_r standards were located by densitometry of the $NaDodSO_4$–polyacrylamide gel and the position of P7P located by autoradiography. The M_r of P7P was estimated by the method of Martin & Ames (1961) assuming a partial specific volume of 0.725 for the standards and P7P. The inclusion of 5 mM 2-mercaptoethanol in the inhibitor buffer did not alter the M_r of P7P.

Miscellaneous Methods. Rabbit muscle cAMP-dependent protein kinase was purified as previously described (Lyons et al., 1975). Thrombin was assayed as previously described by Seegers (Seegers & Smith, 1942). Ca^{2+}–ATPase activity was assayed as previously described (Mendez & Lai, 1975). Protein-bound phosphate was assayed by the method of Ames (1966) or the method of Stull & Buss (1977). [^{14}C]Serotonin release studies were performed as previously described (Tollefsen et al., 1974). PAS staining of $NaDodSO_4$ gels was performed as described by Fairbanks (Fairbanks et al., 1971). Protein was estimated by the method of Lowry (Lowry et al., 1951).

Results and Discussion

Stability Studies. Previously, we demonstrated that $^{32}PO_4^{3-}$, incorporated into P7P as phosphoserine and phosphothreonine, was labile in the presence of *E. coli* alkaline phosphatase (Lyons et al., 1975). Since platelets contain large quantities of both phosphatases (Gordon, 1975) and proteases (Gordon, 1975; Ehrlich & Gordon, 1976; Jakabova & Phillips, 1977; Nachman & Ferris, 1968), it was necessary to examine the stability of the phosphoproteins in platelet homogenates before preceeding with many of the studies described below. When $^{32}PO_4^{3-}$-loaded thrombin-treated platelets were sonicated in resuspension buffer and incubated at 22 °C in the same buffer, there was rapid loss of radiolabel from P7P (Figure 1). This loss was decreased slightly when the sonicate was incubated at 4 °C. The radioactivity of P7P in platelet sonicates could be stabilized by the presence of 0.1 M sodium phosphate, pH 7.4, with approximately 90% of the radioactivity remaining after 75 min at 22 °C. However, after 24 h under the same conditions, only 65% of the radioactivity in P7P remained.

Further studies were undertaken to determine the optimum conditions for long-term stability of P7P in platelet homogenates. $^{32}PO_4^{3-}$-loaded, thrombin-treated platelets were sonicated in inhibitor buffer, incubated at either 4 or 22 °C, and then analyzed for platelet ^{32}P-labeled phosphoproteins by $NaDodSO_4$ gel electrophoresis and autoradiography. The presence of EDTA in this buffer prevented ongoing protein kinase activity. Phosphatases were inhibited by sodium phosphate in combination with β-glycerophosphate and sodium molybdate. Cathepsin D was irreversibly inhibited by 2-mercaptoethanol (Ehrlich & Gordon, 1976) and cathepsin A was competitively inhibited by the addition of the substrate analogue *N*-carbobenzoxy-glycyl-L-phenylalanine (Greenbaum, 1971). Ca^{2+}-activated proteases (Jakabova & Phillips, 1977) were inhibited by EDTA and serine proteases including exogenous thrombin were inhibited by treatment with DFP. No changes were detected in the Coomassie blue staining patterns of the $NaDodSO_4$ gels of the different samples incubated at 4 °C. At 4 °C, over 90% of the radioactivity in P7P remained after 52 h of incubation (Figure 1).

The stability in 11 measureable peaks of radioactivity was similar to that of P7P with the exception of P9P the stability of which was not improved by the presence of inhibitor buffer. P9P lost half of its radioactivity by 4 h at 4 °C and only 27% remained after 20 h of incubation. This inhibitor buffer may be useful for studies of other labile proteins in platelet homogenates.

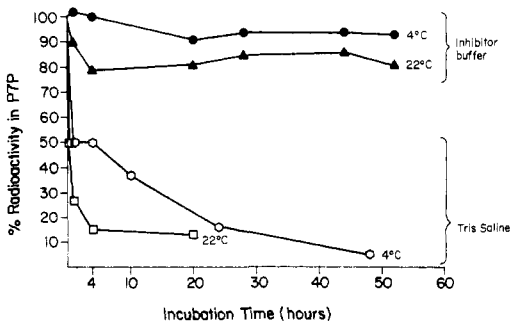

FIGURE 1: Stability of P7P in resuspension buffer or in inhibitor buffer. $^{32}PO_4^{3-}$-loaded thrombin-treated platelets were disrupted by sonication in 10 mM DFP and either resuspension buffer or inhibitor buffer (50 mM sodium phosphate, pH 7.4, 25 mM β-glycerophosphate, 1 mM sodium molybdate, 2 mM EDTA, 0.5 mM *N*-carbobenzoxyglycyl-L-phenylalanine, and 5 mM 2-mercaptoethanol). Aliquots were incubated at either 22 or 4 °C for various times up to 52 h before the reaction was stopped by boiling the platelet protein in $NaDodSO_4$ buffer. The radioactivity in P7P was assessed by $NaDodSO_4$ gel electrophoresis and autoradiography (see legend to Table I) and is presented as a percentage of time zero. (●) Inhibitor buffer at 4 °C; (▲) inhibitor buffer at 22 °C; (○) resuspension buffer at 4 °C; (□) resuspension buffer at 22 °C.

Localization of P7P. Preliminary experiments indicated that P7P was retained during the platelet release reaction. The development of a buffer which stabilized P7P in platelet homogenates allowed us to examine the subcellular location of P7P further. $^{32}PO_4^{3-}$-loaded, thrombin-treated platelets were disrupted by sonication or freeze-thawing in inhibitor buffer as described in Methods, and then separated into soluble and particulate fractions at 4 °C by ultracentrifugation. No difference in P7P distribution was observed between the two methods of platelet disruption. The soluble supernatant contained 79.5% ± SEM 5.3 (N = 5) of the total P7P suggesting an intracellular origin for P7P. Approximately 55% of the total measureable P9P was in the soluble supernatant. However, the interpretation of the latter percentage is difficult since P9P was relatively unstable in platelet sonicates and its stability in the soluble fraction as opposed to the particulate fraction was unknown.

Since it has been demonstrated in other systems that cell disruption can remove membrane associated proteins (Kahan, 1965; Guidotti, 1972), that many phosphate groups are on the platelet surface (Mehrishi, 1970; Stoltz et al., 1975), and that membrane phosphorylation is associated with secretion (Rubin & Rosen, 1975), three other approaches were undertaken in an attempt to determine whether or not P7P was located on the cell surface. Firstly, $^{32}PO_4^{3-}$-loaded platelets were treated with thrombin and then exposed to trypsin as described in Methods in an attempt to hydrolyze surface platelet protein. Trypsin itself induced phosphorylation of P7P and P9P but did not decrease the radioactivity in either P7P or P9P in thrombin-treated platelets (data not shown). Secondly, since lactoperoxidase-catalyzed iodination has been used as a platelet surface probe (Phillips, 1972), we examined the possiblity that P7P or P9P might be iodinated in intact platelets by such treatment, indicating a surface location. Platelets were iodinated either before or after exposure to thrombin. Iodination under the conditions used did not significantly impair thrombin-induced [^{14}C]serotonin release. The pattern of ^{125}I-labeling of the particulate fraction was similar to previously published labeling patterns (Phillips, 1972) (Figure 2). No peaks of radioactivity comigrated with P7P or P9P and no radioactivity was found in the $NaDodSO_4$ gel radiochromatogram of the soluble fraction. Control experiments demonstrated that P7P and P9P could be labeled when lactoperoxidase was added after sonication. Thirdly, to examine the possibility that P7P or P9P in intact platelets might be labeled with an exogenous kinase system (indicating a surface location), we treated intact platelets with [γ-^{32}P]ATP and rabbit muscle protein kinase as described in Methods. Although some labeling of both P7P and P9P was observed, the radioactivity in the two peaks was approximately 5% of that seen under the same conditions using disrupted platelets and thus could be accounted for by the broken platelets usually present in a washed platelet preparation. These experiments indicate that P7P and P9P are not surface proteins.

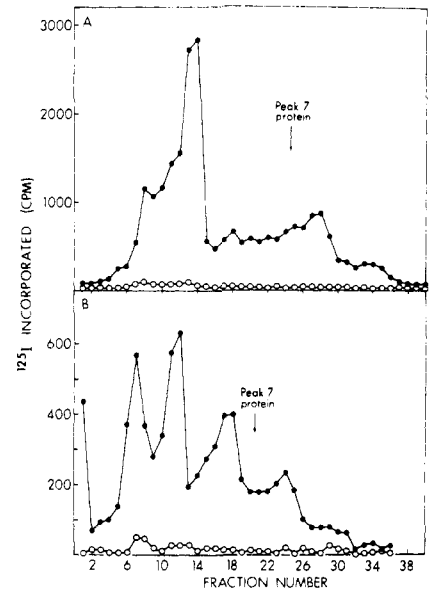

FIGURE 2: $NaDodSO_4$ gel electrophoresis radiochromatogram of the particulate and soluble fractions from intact platelets labeled with ^{125}I by lactoperoxidase-catalyzed iodination. Intact platelets (1 mL at 1 × 10^9 platelets/mL) were labeled with ^{125}I as described by Phillips (1972) either before (A) or after (B) treatment with thrombin I U/mL for 5 min in the presence of 2 mM EDTA. The platelets were washed twice with resuspension buffer, disrupted by sonication in 0.1 M sodium phosphate and 10 mM DFP, and separated into a particulate (●) or soluble (○) fraction by centrifugation at 164000*g* at 4 °C for 60 min. Each fraction was subjected to $NaDodSO_4$ gel electrophoresis on 5% T cylindrical Weber–Osborn gels. The radioactivity in fractions of the stained, destained gels was measured in a γ scintillation spectrometer (Packard). The location of P7P was identified by $NaDodSO_4$ gel electrophoresis of [^{32}P]phosphate-labeled platelet protein in gels electrophoresed in the same experiment. The gels from the experiment depicted in B were slightly shorter than those in A.

Molecular Weight of Peak 7 Protein by Sucrose Density Centrifugation. P7P had a M_r of 47 170 ± SEM 1200 (N = 4) as assessed by sucrose density centrifugation, a value similar to the previously reported M_r of 49 000 as determined by gel filtration (Lyons et al., 1975). No multimeric forms of P7P were seen, supporting our suggestion (Lyons et al., 1975) that P7P is not a subunit of a larger protein.

Molecular Weight of Peak 7 Protein by $NaDodSO_4$ Gel Electrophoresis. We observed that P7P migrated at a different apparent M_r in different $NaDodSO_4$ gel electrophoresis systems. In each of three systems described by Weber & Osborn (1969), Fairbanks (Fairbanks et al., 1971), and Laemmli (1970), P7P migrated in the leading part of the Coomassie blue staining band which also contained actin. The M_r in these systems were 41 450 ± SD 2460 (N = 11), 42 200 ± SD 2070 (N = 20), and 41 800 ± SD 850 (N = 8), respectively. In the system described by Neville (1971) P7P had a significantly higher M_r 48 250 ± SD 2660 (N = 26) (Dunnet, 1964) and migrated above actin.

These findings support preliminary observations that the

mobility of P7P varied in different $NaDodSO_4$ gel electrophoresis systems (Haslam et al., 1977). Similar anomalous mobility has been noted for phosphoproteins in other tissue (Hoffman et al., 1975; Obijeski et al., 1974; Liu & Greengard, 1976). Glycoproteins may migrate variably in different $NaDodSO_4$ gel electrophoresis systems (Maddy, 1976). However, since P7P did not stain in the PAS reaction (see below), it is unlikely that it contains a large amount of carbohydrate. We have been unable to detect either an unusual surface charge density or unusual conformation for P7P[2] and, thus, cannot at this time explain the anomalous migration of P7P on $NaDodSO_4$ gel electrophoresis.

Peak 7 Protein Purification (Table II). For large scale purifications, 60 units of 3–5 day old platelet concentrates were washed as described in Methods, resuspended at a concentration of 6×10^9/mL in resuspension buffer, and incubated with $^{32}PO_4^{3-}$, 0.01–0.02 mCi/mL, for 1 h at 22 °C. The platelet suspension was adjusted to contain 2 mM EDTA and thrombin was added to a concentration of 5 U/mL. After a 5 min incubation at 22 °C, the platelets were sedimented at 3000*g* for 15 min at 4 °C. The supernate was discarded and the pellet was washed once in resuspension buffer containing 2 mM EDTA. The platelet pellet was resuspended in 3 volumes of 6× concentrated inhibitor buffer and the platelets were disrupted by freeze-thawing three times while adjusting the homogenate to contain an additional 2.5 mM DFP during each thawing step. All subsequent steps prior to preparative $NaDodSO_4$ gel electrophoresis were carried out at 4 °C. The entire homogenate was sonicated with the large probe of the Biosonik IV Sonifier at maximum intensity in eight separate 15-s bursts. A final 2.5 mM DFP adjustment was made during sonication for a total concentration of 10 mM. The particulate material was removed by centrifugation at 17700*g* for 10 min followed by further centrifugation of the supernate at 20000*g* for 60 min. The resulting supernate was diluted 1:3 with water and adjusted to pH 4.1 by the dropwise addition of acetic acid, and the precipitate was removed by centrifugation at 17700*g* for 20 min. The second supernate was then harvested and its pH readjusted to 7.4 with NaOH.

Solid ammonium sulfate was added to a final concentration of 30%, the precipitate removed by centrifugation at 17700*g* for 15 min, and the supernate harvested and adjusted to 80% ammonium sulfate. After recentrifugation, the supernate was discarded, and the precipitate dissolved in a small volume of inhibitor buffer and then vacuum dialyzed overnight with three changes against 100 volumes of the same buffer.

The sample was diluted 1:5 with water and immediately passed over a DEAE 52 column which had been equilibrated with a 1:5 dilution of inhibitor buffer. The column was washed with 2 column volumes of the starting buffer and then eluted with a linear gradient from 0 to 0.4 M NaCl containing a 1:5 dilution of inhibitor buffer. Each 5-mL fraction was immediately adjusted to contain 1× inhibitor buffer. The eluate was surveyed by $NaDodSO_4$ gel electrophoresis and the fractions containing P7P, eluting at approximately 0.1 M NaCl, were pooled and concentrated by precipitation with 80% ammonium sulfate.

The ammonium sulfate precipitate was resuspended in a small volume (approximately 5 mL) of inhibitor buffer and vacuum dialyzed overnight against the same buffer containing 10% glycerol. The concentrated protein was incubated at 100 °C for 3 min in Neville stacking buffer containing 1% $NaDodSO_4$ and 0.1 M 2-mercaptoethanol.

[2] R. M. Lyons and R. M. Atherton, unpublished results.

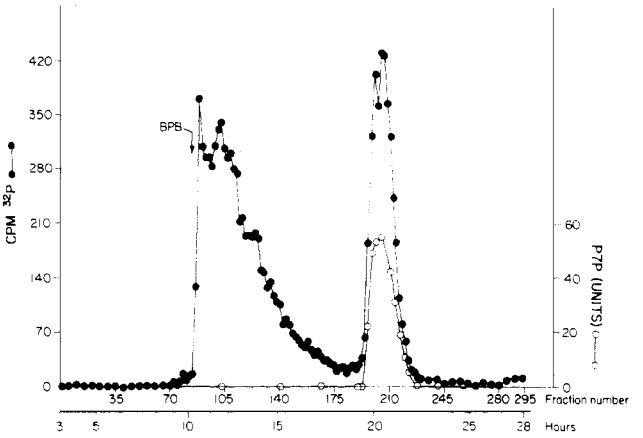

FIGURE 3: Preparative $NaDodSO_4$ gel electrophoresis of [^{32}P]-phosphate-labeled platelet proteins. [^{32}P]Phosphate-labeled platelet proteins, containing P7P, from the DEAE step were fractionated by $NaDodSO_4$ gel electrophoresis on a Buchler Polyprep gel electrophoresis apparatus as described in Methods. The effluent was monitored for [^{32}P]phosphate (●) and for P7P (○) as assessed by analytical $NaDodSO_4$ gel electrophoresis and autoradiography. Bromphenol blue (BPB), indicating the position of the front, was monitored by its absorbance at A_{540}.

The $NaDodSO_4$-treated protein was adjusted to contain 0.003% bromphenol blue and electrophoresed in a Buchler Polyprep 200 preparative gel electrophoresis apparatus at 22 °C as described in Methods. When the constant voltage setting was 50 V until the sample had entered the stacking gel and 100 V thereafter, P7P eluted after approximately 18 h (Figure 3). The location of P7P was identified by the presence of a [^{32}P]phosphate peak in the eluate (Figure 3) and a radioactive band of the appropriate M_r on analytical $NaDodSO_4$ gel electrophoresis (Figures 4A and 4B). Fractions containing P7P free of lower M_r contaminants as assessed by $NaDodSO_4$ gel electrophoresis in the Neville system were pooled and freed of $NaDodSO_4$, sucrose, and nonvolatile salts as described in Methods. The protein was lyophylized and redissolved in a minimal volume of 5 mM sodium phosphate buffer, pH 7.4, 1% $NaDodSO_4$. Two percent of the protein was removed, labeled with fluorescamine as described in Methods, and electrophoresed on a 2.5-mm thick, 10% T Laemmli preparative $NaDodSO_4$ gel electrophoresis slab gel. P7P was cut from the gel, being careful to avoid the higher M_r contaminants, eluted electrophoretically as described in Methods, and then concentrated by lyophylization. The protein was homogeneous as assessed by $NaDodSO_4$ gel electrophoresis in three different systems (Figure 5). This procedure results in a 136-fold purification of P7P with a yield of 3.4% (Table I). These data suggest that P7P comprises approximately 0.65% of the total platelet protein or approximately 8 μg of P7P/10^9 cells. Thus, P7P is a major constituent of the human platelets.

Characterization of Peak 7 Protein. Despite the absence of a positive PAS reaction at the M_r of P7P in $NaDodSO_4$–polyacrylamide gels of total platelet protein (data not shown), it remained possible that P7P, when examined in greater amounts, would give a positive PAS reaction. Thus, 4 μg of homogeneous P7P was electrophoresed by $NaDodSO_4$ gel electrophoresis and stained for carbohydrate by the PAS reaction. No PAS staining took place, suggesting that P7P did not have a high carbohydrate content.

Homogeneous P7P contained 0.3–0.5 mol of phosphate/mol of protein (range of four different measurements, each in duplicate). This is similar to the mol of phosphate/mol of protein reported for other phosphorylated proteins purified in

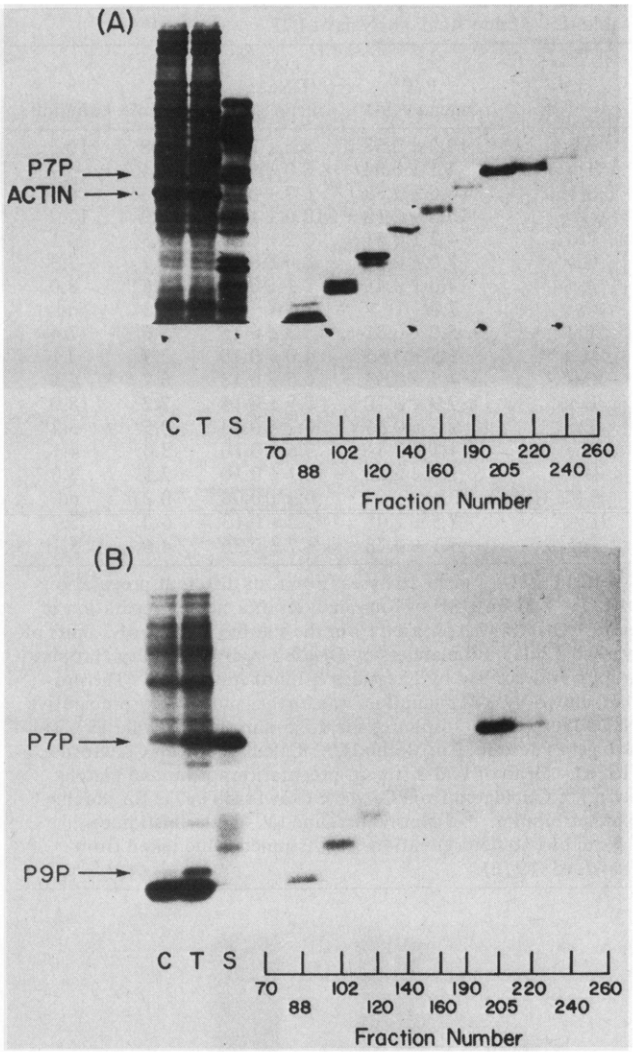

FIGURE 4: Analytical $NaDodSO_4$ gel electrophoresis of fractions from the Polyprep column. Panel A is the Coomassie blue stained pattern and panel B is the autoradiograph of the same gel. C and T are gels of protein from $^{32}PO_4^{3-}$-loaded platelets solubilized in $NaDodSO_4$ buffer either before (C) or after (T) treatment with thrombin, 1 U/mL, for 5 min. S is the starting material. Prior to autoradiography, the filter paper on which the Coomassie stained gel had been dried was marked with radioactive India ink (20 μCi of $H_3{}^{32}PO_4$ in 1 mL of India ink) so that the gel and its autoradiograph could be realigned exactly. This procedure was carried out for all autoradiographs in this study.

buffers containing phosphatase and kinase inhibitors (Nimmo & Cohe ı, 1977). However, it should be viewed as a minimum estimate, since loss of P7P phosphate during the purification procedure was not completely eliminated.

The major isoelectric point of homogeneous P7P was 6.82 ± SEM 0.05 (N = 4) (Figure 6). A minor peak was consistently seen at an isoelectric point of 6.04 ± SEM 0.05 (N = 4). The major isoelectric point of purified P7P was similar to that previously reported by Peterson et al. for P7P in total platelet protein (Peterson et al., 1977).

Table II gives the amino acid composition of P7P. P7P was striking in its content of acidic residues, aspartic acid, and glutamic acid accounting for 23.8% of the total residues. The basic residues, histidine, arginine, and lysine, comprised 14% of the total residues. These data suggest that P7P is an acidic protein. However, because of the neutral isoelectric point measured directly, one must assume that there is considerable amidation of aspartic and/or glutamic acid residues. Of the residues, 30.2% were nonpolar including alanine, valine,

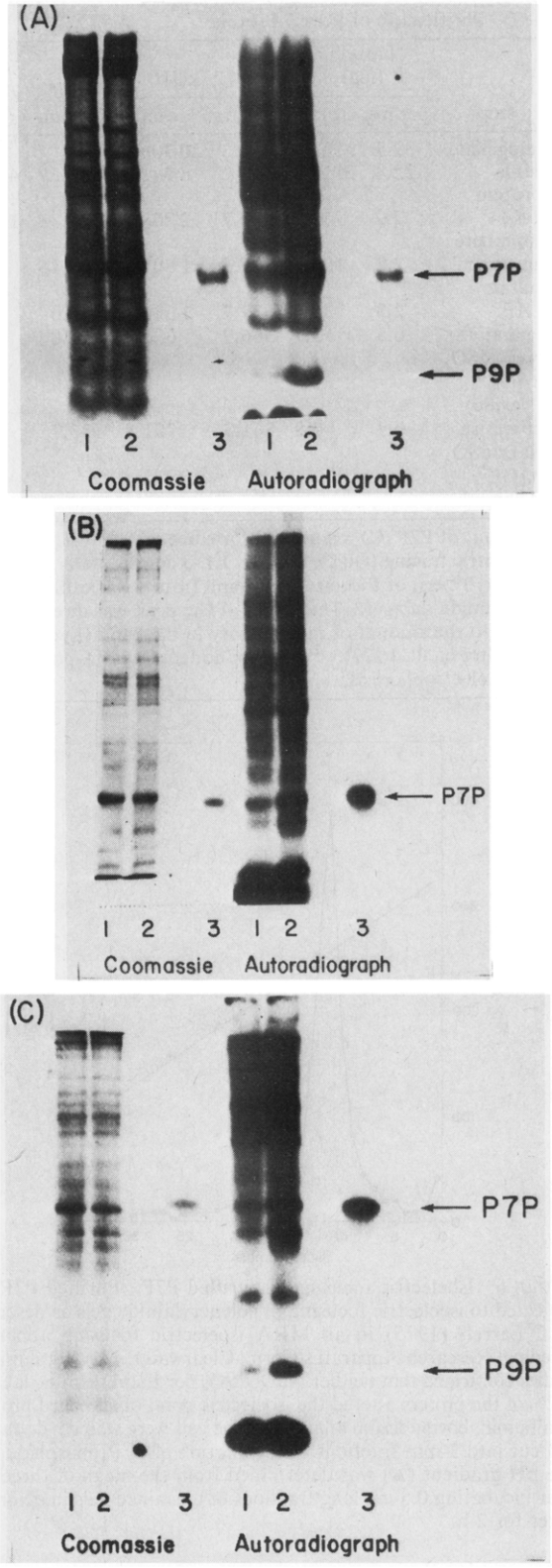

FIGURE 5: Analytical $NaDodSO_4$ gel electrophoresis of purified P7P in three different $NaDodSO_4$ gel electrophoresis systems. (A) Weber–Osborn system 5% T; (B) Laemmli system 8% T; (C) Neville system 13% T. In each panel, gels 1 and 2 are protein from $^{32}PO_4^{3-}$-loaded platelets either before (1) or after (2) exposure to thrombin, 1 U/mL, for 5 min. Gel 3 is purified P7P. In A, P9P migrated in front of the bromphenol blue whose position is indicated by the India ink mark. In (B) P9P migrated at the front. The faint band of radioactivity in B3 seen at the front may represent a small amount of degraded P7P.

methionine, isoleucine, and phenylalanine. When compared with an "average composition of proteins" (Dayhoff et al.,

Table I: Purification of Peak 7 Protein

step	protein (mg) per mL	protein (mg) total	P7P (kU)[a] per mg	P7P (kU)[a] total	fold purification	yield (%)
homogenate	32.8	3287.0	6.3	20 708.1		
soluble protein	22.8	2118.3	4.2	8 981.7	0.67	43.1
pH 4.1 supernate	2.9	253.9	8.7	2 208.9	1.38	10.6
ammonium sulfate	2.2	104.5	17.3	1 807.2	2.75	8.7
DEAE	2.3	35.4	37.7	1 336.2	6.0	6.5
preparative $NaDodSO_4$-PAGE[b] (Neville)	0.3	1.7	366.9	626.0	58.0	3.0
preparative $NaDodSO_4$-PAGE[b] (Laemmli)	0.5	0.8	861.3	701.8	136.7	3.4

[a] One unit of P7P (U) = a peak absorbance of 1.0 at A_{525} on the densitometric tracing (Quick Scan Jr. XL-3 densitometer, Helena, Beaumont, Texas) of the autoradiograph from a $NaDodSO_4$-polyacrylamide slab gel. The height of the peak was directly proportional to the amount of radioactivity in the band (Lyons et al., 1975; Walter et al., 1977). [b] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

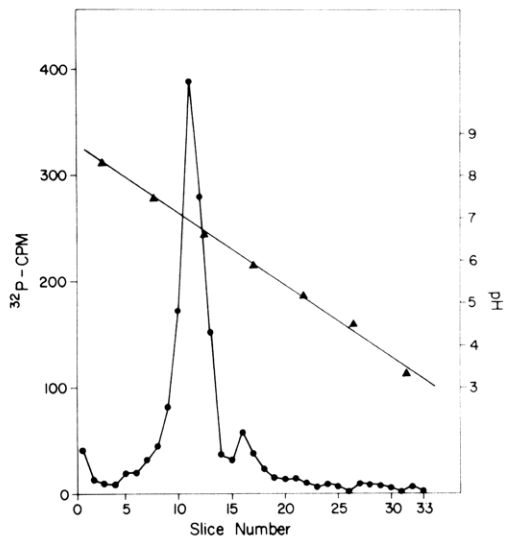

FIGURE 6: Isoelectric focusing of purified P7P. Purified P7P was subjected to isoelectric focusing in polyacrylamide gels as described by O'Farrell (1975) in an MRA isoelectric focusing apparatus (Medical Research Apparatus Corp., Clearwater, FL). Preliminary studies confirmed that neither $NaDodSO_4$ nor fluorescamine labeling of 2% of the protein altered the isoelectric point of standard proteins (ovalbumin, bovine serum albumin). The gels were stained, destained, and cut into 2-mm fractions for evaluation of [^{32}P]phosphate (●). The pH gradient (▲) was determined from the mean of three gels after incubating 0.5-cm long fractions of unstained gels in deionized water for 2 h.

Table II: Amino Acid Analysis of P7P

	P7P[a] mean ± SD	actin[b] (DNase I) mean ± SD	actin[c]	tubulin[d]
Asx	10.0 ± 0.58	8.5 ± 0.13	8.8	10.3
Thr	4.1 ± 0.94	6.8 ± 0.10	6.4	5.7
Ser	6.6 ± 2.57	6.7 ± 0.74	6.8	6.6
Glx	13.8 ± 2.18	12.0 ± 1.27	10.9	12.2
Pro	4.2 ± 0.51	5.2 ± 0.25	5.5	6.1
Gly	7.0 ± 0.51	8.0 ± 0.17	7.1	7.9
Ala	7.0 ± 0.45	8.1 ± 0.19	7.6	8.0
Cys	7.8[f]	(1.3)[g]	1.3	nd
Val	5.0 ± 0.51	5.3 ± 0.15	5.6	7.4
Met	1.5 ± 0.55	4.0 ± 0.10	3.5	2.1
Ile	4.7 ± 0.65	6.6 ± 0.18	6.7	4.8
Leu	7.9 ± 0.70	7.9 ± 0.15	7.2	8.0
Tyr	2.3 ± 0.55	4.0 ± 0.13	3.5	3.2
Phe	4.1 ± 0.84	3.6 ± 0.10	3.6	4.1
His	1.4 ± 0.16	2.1 ± 0.10	2.1	3.0
3-Me-His[e]	0	0.2 ± 0.06	0.27	nd
Lys	7.5 ± 1.0	5.2 ± 0.13	6.0	5.7
Arg	5.1 ± 0.78	4.7 ± 0.36	4.6	5.1

[a] Mean ± SD of eight analyses from four different preparations of P7P. [b] Mean ± SD of four analyses of a single preparation of actin. G-actin was purified from the soluble portion of 3 units of washed 72 day old platelets by DNase I-agarose affinity chromatography as described by Lazarides & Lindberg (1974). The protein, eluted by 3 M guanidine, was further purified by preparative $NaDodSO_4$ gel electrophoresis in a 2.5-mm thick Neville 13% T slab gel as described in Methods. [c] Calculated from Crawford (1976). Mean of two different preparations of human platelet actin. [d] Calculated from Castle & Crawford (1977) for porcine platelet tubulin. [e] 3-Methylhistidine (N^{τ}-methylhistidine). [f] Mean of two determinations. [g] Assumed value taken from Crawford (1976).

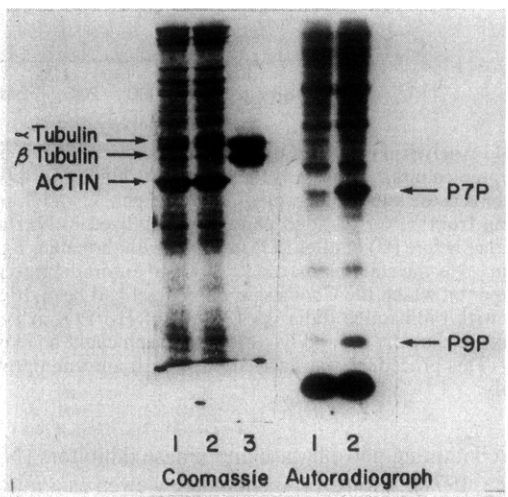

FIGURE 7: Electrophoresis of ^{32}P-labeled platelet proteins and homogeneous bovine renal cortical tubulin on a 10% T Laemmli $NaDodSO_4$–polyacrylamide slab gel. Gel 1 is $^{32}PO_4^{3-}$-loaded control platelets; gel 2 is $^{32}PO_4^{3-}$-loaded thrombin-treated platelets; gel 3 is 5 μg of bovine renal cortical tubulin. The two subunits of tubulin are indicated as α and β.

1976), only four amino acids in P7P varied by more than 30% from the average, with glycine and cysteine above and tyrosine and histidine below the average. A low tyrosine content has been noted in other proteins purified by $NaDodSO_4$ gel electrophoresis (Weber & Osborn, 1975). However, we did not observe this phenomenon in actin prepared by $NaDodSO_4$ gel electrophoresis (Table II).

Relationship of P7P to Actin. Both actin and P7P migrate in the same dark Coomassie blue staining region on Weber–Osborn $NaDodSO_4$ gels. However, P7P did not coelectrophorese with actin on Neville $NaDodSO_4$ gels and did not copurify with actin purified as actomyosin by salt extraction (Adelstein et al., 1973) or as G-actin by DNase I affinity chromatography (data not shown). These data are similar to those published by others (Daniel et al., 1977; Haslam & Lynham, 1976) suggesting that P7P is not actin. These data are not conclusive since it is possible that phosphorylated actin or a form of actin (Probst & Lüscher, 1972; Abramowitz et al., 1975) might behave in a very different manner from nonphosphorylated actin in any or all of the experiments described above.

The amino acid composition of homogeneous actin purified

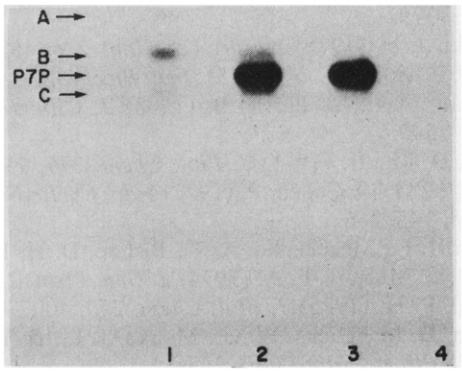

FIGURE 8: Autoradiograph of a 10% T Laemmli $NaDodSO_4$ gel electrophoresis showing the migration of homogeneous P7P and soluble platelet protein labeled with 8-N_3-[^{32}P]cAMP. Photoaffinity labeling was performed by a modification of the method of Walter (Walter et al., 1977). Platelets from fresh blood were washed as described above and resuspended in resuspension buffer at a concentration of 6×10^9/mL. After exposure to thrombin (1 U/mL for 5 min), the suspension was sonicated and adjusted to contain 10 mM DFP and inhibitor buffer. The soluble protein was harvested after centrifugation at 200000*g* for 45 min at 4 °C and 0.5 mL was passed over a 1 × 21.5 cm column of Sephadex G-25 equilibrated with inhibitor buffer. The protein in the void volume was concentrated to 0.6 mg/mL by vacuum dialysis. The final reaction mixture of 40 μL consisted of 15 μg of protein, 1 mg/mL of theophylline, 10 μM adenosine, and 0.4 μM 8-N_3-[^{32}P]cAMP with or without 10 μM cAMP added 10 min before the 8-N_3-[^{32}P]cAMP. After a 1-h incubation at 4 °C in the dark, the samples were irradiated for 10 min at 254 nm with a Mineralight hand lamp at a distance of 8 cm and the samples adjusted to contain $NaDodSO_4$, heated, and electrophoresed on a $NaDodSO_4$ slab gel. A, B, and C indicate the major 8-N_3-[^{32}P]cAMP-labeled bands. (Gel 1) Platelet soluble protein labeled with 8-N_3-[^{32}P]cAMP. (Gel 2) 8-N_3-[^{32}P]cAMP-labeled protein mixed with homogeneous P7P. (Gel 3) Homogeneous P7P alone. (Gel 4) 8-N_3-[^{32}P]cAMP, 0.4 μM and cAMP, 10 μM, added 10 min prior to the 8-N_3-[^{32}P]-cAMP.

by DNase I affinity chromatography and preparative $NaDodSO_4$–gel electrophoresis was similar to the previously published amino acid analysis of human platelet actin (Table II). P7P differed significantly from actin in 10 of 16 amino acids where enough data were available to apply Student's *t* test (Snedecor & Cochran, 1967). The amino acid analyses of all actins, including platelet actin, are characterized by the presence of 3-methylhistidine, with human platelets containing 0.88–1 mol of 3-methylhistidine/42 000 g of protein (Crawford, 1976). Actin in this study contained 0.203 mol of 3-methylhistidine/100 residues or 0.75 mol of 3-methylhistidine/42 000 g of protein. P7P did not have any detectable 3-methylhistidine even when the sample load on the amino acid analyzer was increased so that less than 0.1 mol of 3-methylhistidine/mol of protein could have been detected. These data conclusively indicate that P7P is not a phosphorylated form of actin.

Relationship of P7P to Tubulin. We considered it possible that P7P might be the phosphorylated β subunit of tubulin (Pipeleers et al., 1977; Ikeda & Steiner, 1976; Snyder & McIntosh, 1976; Castle & Crawford, 1976). However, P7P migrated well below the β subunit of highly purified bovine renal cortex tubulin in Laemmli $NaDodSO_4$ gel electrophoresis (Figure 7). Furthermore, the published amino acid analysis of pig platelet tubulin (Table II) (Castle & Crawford, 1977) differed from P7P by 20% in 8 of the 16 residues compared. These data strongly suggest that P7P is not tubulin.

Relationship of P7P to the Regulatory Subunits of the cAMP-Dependent Protein Kinases. It was possible that P7P was the phosphorylated regulatory subunit of a type II cAMP-dependent protein kinase (Hoffmann et al., 1975; Lyons et al., 1975; Salzman & Weisenberger, 1972; Kaulen & Gross, 1974; Kahlé et al., 1975; Booyse et al., 1976). Thus, cAMP-binding proteins in soluble protein from thrombin-treated platelets were linked covalently to 8-N_3-[^{32}P]cAMP by photoaffinity labeling and their mobility was compared with that of P7P by $NaDodSO_4$ gel electrophoresis (Figure 8). No 8-N_3-[^{32}P]cAMP band coelectrophoresed with P7P, suggesting that P7P is not a regulatory subunit of a cAMP-dependent protein kinase.

A review of phosphorylated proteins involved in regulatory mechanisms that have been reported in the literature (Rubin & Rosen, 1975; Nimmo & Cohen, 1977; Langan, 1973) does not reveal a phosphoprotein with physicochemical characteristics (M_r, isoelectric point, amino acid analysis) similar to those of P7P. Nonetheless, since few of these phosphoproteins have been well characterized in platelets, it remains possible that P7P has a function similar to that of a known phosphoprotein. This suggestion will be tested when P7P becomes available in a nondenatured form. However, at the present time, the identity and function of this major soluble platelet phosphoprotein, and its possible role in the platelet release reaction, remain unknown.

Acknowledgments

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