Solubilization and Characterization of a Platelet Membrane ADP-binding Protein*

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Previous studies have shown that platelet membranes bind radiolabeled ADP and have nucleoside diphosphokinase activity which transforms added ADP to ATP. In order to further characterize these reactions, the ADP-binding and nucleoside diphosphokinase activity of purified platelet membranes were solubilized by freeze-thaw injury followed by extraction with isotonic buffered saline. Up to 80% of membrane ADP-binding activity was solubilized along with 20% of the total membrane protein, a 4-fold purification. A Millipore filter binding assay was developed to detect the soluble binding protein using [3H]ADP as radioligand. Binding of [3H]ADP was rapid, reversible, saturable, and was destroyed by heat, trypsin digestion, and 1 mM N-ethylmaleimide. By Scatchard analysis, there was a single class of binding sites with a K_d of 3.8 x 10^-7 M. Unlabeled nucleotides competed with [3H]ADP with the following potency series: ATP = ADP > AMP >> adenosine. The solubilized nucleoside diphosphokinase activity could be separated from ADP-binding activity by ultracentrifugation on 5 to 20% sucrose density gradients containing 0.6 M KCl suggesting that the activities reside on separate molecules. Hydrodynamic parameters were calculated for the binding protein by gel filtration and ultracentrifugation. The S_value was 4.1, Stoke's radius 35 x 10^-8 cm, axial ratio (f/h) 1.09, and the M_(o) = 81,000. The studies suggest that this platelet ADP-binding protein may act as the receptor for initiating ADP-induced aggregation and release.

An alternate view is that there are ADP receptor proteins on the platelet surface which are separate from the ADP-metabolizing enzymes and that the binding of ADP to these sites initiates aggregation. These receptors would then be similar to platelet membrane receptors that have been described for thrombin (5) and epinephrine (6). Born was the first to measure ADP binding to intact platelets and to propose the existence of such an ADP receptor (7). Numerous investigators have subsequently analyzed the binding of ADP to platelets with estimates of 80,000 to 200,000 binding sites/platelet (8-10). Naculau and Ferisi then demonstrated saturable, reversible binding to a plasma membrane fraction derived from platelets (11). These studies with intact platelets and membrane fractions have not fully resolved the two mechanisms proposed for aggregation and have not determined whether ADP binding is independent of enzymatic transformation. In this paper, we report the solubilization and characterization of platelet membrane ADP-binding sites and their resolution from platelet enzymes that metabolize adenosine nucleotides.

MATERIALS AND METHODS

Chromatography Media—Sephadex G-25, Sepharose 2B, 6B, and blue dextran were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Avicel cellulose precoated thin layer chromatography plates were obtained from Analtech, Inc., Newark, Del. Dowex 50W-X8 was purchased from Bio-Rad Laboratories, Rockville Center, N. Y. A type HA 0.45 μ Millipore membrane, Bedford, Mass., was used.

Chemicals and Nucleotides—Acrylamide, methylenebisacrylamide, N,N',N'-tetramethylethylenediamine, and sodium dodecyl sulfate were purchased from Eastman Kodak Co., Rochester, N. Y. Triton X-100 was obtained from George Mann Co., Stoneham, Mass. o-Nitrophenyl-β-D-galactopyranoside-1-diphosphate, pyruvic acid, NADH, L-malic acid, AMP, ADP, ATP, and N-ethylmaleimide were purchased from Sigma Chemical Co. All other chemicals were reagent grade. The nucleotides were analyzed by high voltage electrophoresis and were 95% pure by this technique. For some experiments the ATP was repurified on Dowex 50W immediately prior to use.

Proteins—Trypsin/tosylphenylalanyll chloromethyl ketone was purchased from Worthington Biochemical Corporation, Freehold, N. J. Pumaresa, β-galactosidase, cytochrome c, catalase, lactate dehydrogenase, and nucleoside-5'-diphosphate kinase were obtained from Boehringer Mannheim, New York. Crystalline bovine serum albumin was purchased from Nutritional Biochemicals, Cleveland, Ohio. Human hemoglobin was obtained by lysis of red blood cells from normal volunteers as described by Drabkin (12). 125I-labeled human serum albumin was obtained from Mallinkrodt Chemical Works, New York.

Radioactive Nucleotides—[8-3H]Adenosine 5'-diphosphate ([3H]-ADP, 13 to 16 Ci/mmole) and [8-3H]Adenosine 5'-triphosphate ([3H]-ATP, 12 Ci/mmole) were purchased from Schwarz/Mann, Orangeburg, N. Y. [3H]Adenosine 3',5'-monophosphate ([3H]AMP, 30 Ci/mmole) was obtained from New England Nuclear.

Platelets—Normal human platelets, anticoagulated with acid citrate dextrose (NIH formula A) and obtained within 72 h of collection were kindly provided by the Massachusetts Division of the American Red Cross. A unit of platelets contained an average of 5 x 10^10 platelets in 30 ml of plasma. Platelets were counted with an electronic...
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Particle Counter (Coulter model F, Coulter Electronics, Hialeah, Fla.)

Buffers—Krebs Ringer phosphate buffer (KRP buffer) contained 0.15 M NaCl, 0.01 M PO₄³⁻, 0.001 M MgCl₂, 0.001 M CaCl₂, and 0.005 M KCl, pH 7.4. Tris/saline buffer (pH 8.2) contained 0.01 M Tris-HCl and 0.15 M NaCl.

Platelet Membrane Isolation—Platelet concentrates containing 2 to 4 x 10⁹ platelets/ml of plasma were centrifuged at 37°C for 10 min to sediment contaminating red blood cells. The supernatant was removed and centrifuged at 7,800 x g for 10 min. The platelet pellet was resuspended in the KRP buffer containing a concentration of 4 x 10⁴ platelets/ml. Membrane isolation was carried out according to the procedure of Barber and Damies (16) with the following variations. The platelet lysate was centrifuged on a one step 27% sucrose density gradient at 35,000 rpm (170,000 x g) for 2 h at 4°C in a Beckman model L5-65B ultracentrifuge equipped with an SW 40 rotor. The platelet membrane suspension was recovered from the buffer-sucrose interface and recentrifuged at 40,000 rpm (210,000 x g) for 45 min at 4°C. The platelet membrane pellet was then suspended in KRP buffer.

[¹H]ADP Binding—Variable quantities of [¹H]ADP were incubated with between 40 and 80 µg of platelet membrane or solubilized protein suspended in either Tris/saline or KRP buffer at 37°C. Following the incubation period, the samples were filtered through 0.45-µm Millipore filters under reduced pressure. The filters were then washed with buffer and the activity on the filters and the adsorbed radioactivity was measured in a liquid scintillation spectrometer with an overall efficiency of 40% (Searle Analytic, Des Plaines, Ill.). In addition, parallel incubations were carried out containing tracer and 1 µM nonradioactive ADP to determine nonspecific binding. To determine the amount of [¹H]ADP which was bound directly to the membrane protein, samples were incubated in the absence of any platelet membrane protein. Filter blank and nonspecific binding were subtracted from values obtained for total binding.

Protein Measurement—Protein concentration was estimated by the method of Lowry et al. (14) using bovine serum albumin as a standard. When assaying protein solutions containing Triton X-100, the protein-free precipitate that formed was pelleted by centrifugation at 100,000 x g for 10 min before reading absorbance at 280 nm.

Solubilization of [¹H]ADP Binding Activity—Five successive Triton X-100 extractions were performed on a 1.7-mg sample of platelet membranes suspended in 0.5 ml of Tris/saline. The Triton X-100 concentration was progressively increased from 0.2 to 2% and the membranes were incubated at 37°C with continuous stirring for 2 h with each concentration of detergent. Following each incubation, the mixture was ultracentrifuged at 50,000 rpm (300,000 x g) in a Beckman SW 50.1 rotor for 45 min at 4°C. The 0.6-Ml supernatant was removed and its protein concentration was estimated. The insoluble pellet was then resuspended in 0.6 ml of the next highest detergent concentration and the extraction procedure was repeated.

Preparation of Red Cell Membrane—Two milliliters of packed red cells were centrifuged at 100,000 x g for 30 min in a sucrose-phosphate-saline (0.15 M KCl, 0.05 M PO₄⁻, pH 8.0) buffer containing 0.15 M NaCl. The cells were then lysed in 10 to 20 volumes of 0.05 M PO₄⁻ buffer (pH 8.0) as described by Dodge et al. (16). Red cell membrane vesicles were then pelleted by spinning in a Sorvall RC-3 centrifuge at 20,000 rpm for 10 min. The pellet was washed 4 times in 0.005 M PO₄⁻ buffer. After the final wash, the membrane pellet was resuspended in KRP buffer and its protein concentration was determined.

Method for Assaying Nucleoside Diphosphokinase Activity—A 50-µl aliquot of the sample to be analyzed was incubated at 37°C for 5 min in a system containing 100 pmol of [¹H]ADP and either ATP or GTP (80 µM). The 75-µl mixture was buffered with KRP buffer. Following the incubation, 100 µl of 5% perchloric acid, cooled to 4°C, were added to each sample. The samples were centrifuged at 7000 x g for 10 min at 4°C, after which 100 µl of each supernatant were collected. Then 10 µl of 3 M K₂CO₃ were added to each of the samples, which were then centrifuged again for 10 min at 7000 x g. Ten microliters of supernatant (approximately 1.0 pmol of radioactive nucleotide) were removed from this final mixture and its adenine nucleotide composition was analyzed by high voltage electrophoresis.

Analysis of Adenine Nucleotide Composition Using High Voltage Electrophoresis on Cellulose Thin Layer Plates—All samples to be analyzed were applied to an Avian cellulose thin layer chromatography plate (20 x 40 cm). A 5-µl aliquot containing 20 pmol each of nonradioactive ADP and ATP was applied to those spots to which the samples had been previously adsorbed. The samples were then subjected to electrophoresis on a cooling plate (Savant Instrument, Inc., Hicksville, N. Y.) for 100 min at 1000 V with a buffer made up of 0.05 M potassium acid tiritated with 0.08 M sodium tiritate to pH 3.8. Following electrophoresis, the plates were air-dried. Nucleotide spots corresponding to ADP and ATP were located with a short wave ultraviolet light and scraped from the plate. The adsorbed radioactivity was eluted from the cellulose material with 0.5 ml of distilled water. The radioactivity in a 100-µl aliquot of the eluate was then determined in a liquid scintillation counter. On an average, samples containing approximately 1.0 pmol of radioactive nucleotide were electrophoresed and 90% of the applied radioactivity was recoverable as either ADP or ATP.

Sucrose Gradient Ultracentrifugation—4.6 ml of 5 to 20% sucrose gradients were prepared according to the method of Martin and Ames (17) on a Spinco LSE ultracentrifuge. The samples were then applied to either KRP buffer or KRP buffer containing 0.6 M KCl. Protein samples, in 200-µl volumes, were layered on the top of the gradient ultracentrifuged at 50,000 rpm (300,000 x g) in an SW 50.1 rotor for 17 h at 4°C. After centrifugation, 12 to 15 8-drop fractions were collected from the bottom of the tube.

Sepharose 6B Gel Filtration—A column of Sepharose 6B (1.2 x 90 cm) was equilibrated and eluted with KRP buffer containing 0.6 M KCl, pH 7.6, at 4°C. The void volume (34.2 ml) was determined by the elution position of blue dextran 2000. The sample was applied in a 1.2-ml volume and 1.8-ml fractions were collected. In these studies, β-galactosidase, lactate dehydrogenase, fumarase, catalase, hemoglobin, and β-galactosidase were used as standard proteins. All the marker enzymes were applied to the column along with the KRP buffer. Hemoglobin and glucose-6-phosphate dehydrogenase were chromatographed separately because they interfered with the [¹H]ADP binding assay. β-Galactosidase and catalase were included in both filtration experiments to ensure that the data from the two columns were comparable. Hemoglobin was detected in the column fractions by its absorbance at 540 nm. ¹H-albumin was detected by counting 100-µl aliquots from the fractions in a liquid scintillation counter.

Enzyme Assays—All enzyme assays were conducted at room temperature, on a Gilford 2400 spectrophotometer (Gilford Instrument, Oberlin, Ohio) as described by Neer (18). Unless otherwise noted, substrates were buffered in 0.02 M phosphate, pH 7.6. Lactate dehydrogenase was assayed by the decrease in absorbance at 340 nm in 0.22 M pyruvic acid and 0.001 M sodium pyruvate in 1.0 M NaCl. Enzyme activity was measured by the increase in absorbance at 240 nm in 0.05 M L-malic acid. Catalase consumption of hydrogen peroxide was monitored at 240 nm in 16 mM H₂O₂. β-Galactosidase activity was detected in 1.2 mM o-nitrophenyl galactoside at 400 nm, buffered in 0.05 M Tris pH 7.6. For each assay, absorbance was recorded at 15-s intervals, and enzyme activity was calculated from the initial portion of the time versus absorbance curve.

RESULTS

Solubilization of [¹H]ADP Binding Activity—For these studies, a sample of frozen and thawed platelet membrane was extracted with increasing concentrations of Triton X-100 and the remaining membrane material pelleted by ultracentrifugation. When the supernatants were assayed for soluble [¹H]ADP binding activity using the Millipore filtration technique, 65% of the original activity had been solubilized by the initial extraction with Tris/saline buffer (Table I). This Tris/saline extract bound 67 pmol of [¹H]ADP/mg of protein. Subsequent extraction with Triton X-100 yielded less total [¹H]ADP binding activity with a lower specific activity.

After these initial studies, it was observed that the amount of [¹H]ADP bound by the soluble protein could be increased by 150% when KRP buffer was substituted for Tris/saline buffer in the extraction and assay procedures. Consequently, KRP buffer was used in subsequent membrane extractions.
and in the \( ^3\)HADP binding assay. When KRP buffer was used to extract a sample of repeatedly frozen and thawed platelet membrane, nearly 60% of all recovered \( ^3\)HADP binding activity was solubilized and present in the supernatant with a specific activity of approximately 153 pmol of ADP/mg of protein. For comparison, the platelet membranes bound 85 pmol of \( ^3\)HADP/mg of protein at saturation. Thus, the supernatant from the extracted pellet contained almost 80% of the \( ^3\)HADP binding activity present in platelet membranes. Approximately 20% of the total protein was also solubilized. Consequently, the KRP buffer extraction procedure resulted in a 4-fold purification of \( ^3\)HADP binding activity. It was noted that subsequent extractions of the insoluble pellet with KRP buffer solubilized additional small quantities of \( ^3\)HADP binding activity. Moreover, some binding activity was also observed in the sucrose gradients on which platelet membrane isolation had been performed. The \( ^3\)HADP binding activity of the KRP buffer-solubilized platelet membrane was stable over a period of 3 months when stored at \(-20^\circ\)C.

An analysis of the KRP buffer extract using sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the solution to be a heterogeneous mixture of proteins. When a 45-\( \mu \)g aliquot of the membrane extract was subjected to electrophoresis on a 5% acrylamide gel containing 0.1% sodium dodecyl sulfate, approximately 10 bands were visible after staining with Coomassie blue (not shown).

**Kinetics of \( ^3\)HADP Binding to the KRP Buffer Extract**—

The effect of temperature on the interaction of \( ^3\)HADP with the KRP buffer extract is depicted in Fig. 1. While 50% of maximal binding was achieved after 1 min of incubation at \(37^\circ\)C, it required 3 min of incubation at \(25^\circ\)C to achieve this same degree of binding. Similarly 22 min of incubation at \(0^\circ\)C were required for the sample to achieve 50% of maximal binding.

\( ^3\)HADP bound to the KRP buffer extract was displaced by an excess of unlabeled ADP. In a time-dependent dissociation study (Fig. 1), 1 h after the addition of nonradioactive ADP to the \(37^\circ\)C incubation system, 92% of the original KRP buffer extract bound \( ^3\)HADP had been dissociated.

The binding of \( ^3\)HADP was also saturable. Analysis of the data by the method of Scatchard showed that there was a single class of binding sites which had a dissociation constant \((K_d)\) of \(3.8 \times 10^{-7}\) M (Fig. 2). The KRP buffer extract bound

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bound ( ^3)HADP</th>
<th>Recovered ( ^3)HADP binding</th>
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</thead>
<tbody>
<tr>
<td>Platelet membrane suspension (0.5 ml)</td>
<td>0.78</td>
<td>100</td>
</tr>
<tr>
<td>Saline extract</td>
<td>0.39</td>
<td>60</td>
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<tr>
<td>0.1% Triton X-100</td>
<td>0.03</td>
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<tr>
<td>0.5% Triton</td>
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<td>17</td>
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<td>05</td>
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<tr>
<td>2% Triton</td>
<td>0.02</td>
<td>03</td>
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<tr>
<td>&gt;2% Triton</td>
<td>0.02</td>
<td>03</td>
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<tr>
<td>Total recovery</td>
<td>36</td>
<td>92</td>
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$\text{Recovery} = \frac{\text{Bound pmol}}{\text{Recovered pmol}} \times 100$

$\text{% original} = \frac{\text{Bound pmol}}{\text{Recovered pmol}} \times 100$

$\text{Protein concentration} = \frac{\text{Bound pmol}}{\text{Recovered pmol}} \times 100$

$\text{Specific activity} = \frac{\text{Bound pmol}}{\text{Recovered protein}}$
Adenine Nucleotide Inhibition of [³H]ADP Binding—-binding experiments were conducted, as shown in Fig. 3, in which increasing amounts of nonradioactive adenine nucleotide were incubated with the KRP buffer extract along with [³H]ADP. ADP was the most potent inhibitor of [³H]ADP binding. While AMP demonstrated some ability to compete for [³H]ADP binding sites on membrane extract, adenosine did not inhibit binding at any concentration tested. ATP was as potent an inhibitor of this interaction as was ADP. Purification of the ATP by ion exchange chromatography on Dowex 50 just prior to incubation did not alter its ability to block [³H]ADP binding. In addition, the extract was assayed for its ability to directly bind [³H]AMP using the Millipore technique. When [³H]AMP was incubated with an aliquot of KRP buffer-solubilized platelet membranes, the maximal binding measured was less than 1 pmol/mg of protein.

[³H]ADP Binding to Red Cell Membranes and Other Proteins—These studies sought to evaluate whether the interaction of ADP with platelet membranes or solubilized membrane proteins was a unique phenomenon. When [³H]-ADP was incubated with suspensions of sonicated red blood cell membranes, approximately 2.1 pmol of [³H]ADP/mg of membrane protein were collected on the Millipore filter discs (Table III). However, 40% of the radioactivity absorbed to the filter could not be displaced by up to 1 mM unlabeled ADP, suggesting that it might be trapped nonspecifically within disrupted membrane vesicles. A more accurate estimate of specific binding of the red blood cell ghosts is 1.3 pmol of [³H]ADP bound/mg of protein. In addition to red blood cell membranes, hemoglobin, albumin, and nucleoside diphoshokinase, an enzyme which uses ADP as a substrate, were tested for the capacity to bind [³H]ADP by the Millipore technique (Table III). Of the three proteins, hemoglobin had the greatest capacity for [³H]ADP, with a specific activity of 0.8 pmol of nucleotide bound/mg of protein at saturation.

Conversion of ADP to ATP by the KRP Buffer Extract—In these studies, [³H]ADP was incubated with aliquots of KRP buffer-solubilized platelet proteins along with nonradioactive ATP or GTP. Following the incubation, the adenine nucleotide composition was analyzed by high voltage electrophoresis on cellulose thin layer plates. When ATP was included in the incubation system, nearly 90% of the original [³H]ADP was converted to [³H]ATP after a 15-min incubation with a sample of KRP buffer extract (Fig. 4). However, in the absence of any high energy phosphate donor, [³H]ADP was not metabolized. After a brief lag period, the rate of conversion was linear during the 15 min of incubation.

Ultracentrifugation of KRP Buffer Extract on Sucrose Gradients—In these experiments, samples of KRP buffer membrane extract were ultracentrifuged on 5 to 20% sucrose gradients. The ADP-binding protein was localized by assaying aliquots from each fraction for binding activity. Cytochrome c, hemoglobin, and 125I-albumin were centrifuged in separate gradients and served as calibrating proteins. When this procedure was carried out in an ionic environment similar to KRP buffer (0.15 M NaCl, 0.005 M KCl), ADP binding activity was distributed throughout the high density fractions of the gradient (Fig. 5). However, when the experiment was repeated in a gradient containing 0.6 M KCl, binding activity was

| Table II |
| Pretreatment | [³H]ADP bound pmol/mg protein | Initial activity % |
| None | 97 | 100 |
| 10⁻⁴ M N-Ethylmaleimide | 70 | 72 |
| 10⁻³ M N-Ethylmaleimide | 35 | 36 |
| 10 µg/ml trypsin | 40 | 41 |
| 100°C for 5 min | 1.3 | 1 |

Table III

| Protein | [³H]ADP bound pmol/mg protein % |
| KRP membrane extract | 153 | 100 |
| Red blood cell membrane | 2.1 | 1.4 |
| Bovine serum albumin | <0.1 | <0.1 |
| Nucleoside diphosphokinase | 0.4 | 0.9 |
| Human hemoglobin | 0.8 | 0.5 |

Fig. 3. Inhibition of [³H]ADP binding to KRP buffer extract by adenine nucleotides. Various adenine nucleotides, at the specified concentrations, were incubated with 5 µg of KRP buffer-solubilized platelet membrane for 30 min at 37°C along with 22 pmol of [³H]-ADP. The indicated adenine nucleotide concentrations refer to their final concentration in the 75 µl incubation. All samples were filtered and processed as described in the text and Fig. 1. [³H]ADP binding is presented as a percentage of the radioactivity bound when no other adenine nucleotides were added to the incubation system. This 100% control sample was run in triplicate and averaged. ATP was purified by ion exchange chromatography, as described under "Materials and Methods," just prior to use. The adenine nucleotides studied were: ●, adenosine; ■, AMP; ○, ADP; △, ATP.

Fig. 4. Of KRP buffer extract 6 µg were incubated with 0.3 nmol of [³H]ADP in 0.5 ml of KRP buffer at 37°C. At intervals, 50-µl aliquots, representing 30 pmol of radioactive nucleotide, were removed from the incubation mixture and added to 100 µl of 5% perchloric acid, cooled to 4°C. The adenine nucleotides were extracted and their compositions were analyzed as described in the text. Approximately 90% of the 1.0 pmol of radioactive nucleotide applied to the thin layer plate were recovered as either [³H]ADP or [³H]ATP after high voltage electrophoresis. Picomoles of [³H]ATP formed refer to the amount of radioactivity that migrated on the plate with nonradioactive carrier nucleotide. Values represent the mean of duplicate experiments.
located in a discrete peak (Fig. 6). On the basis of the distance sedimented by the [3H]ADP binding protein, relative to that of the standard proteins, a sedimentation coefficient of $s_{20,w} = 4.1$ S was calculated.

**Separation of Nucleoside Diphosphate Kinase and [3H]-ADP Binding Activities**—For these studies, a 5 to 20% sucrose gradient containing 0.6 m KCl, on which KRP buffer extract had been ultracentrifuged, was assayed for both nucleoside diphosphate kinase and [3H]ADP binding activities. Enzyme activity peaked in the gradient fraction whose corresponding sedimentation coefficient was approximately $s_{20,w} = 5$ S (Fig. 6). The gradient fractions were also analyzed for [3H]ADP binding activity. The nucleoside diphosphate kinase and ADP binding peaks were clearly separable (Fig. 6).

**Separase 6B Gel Filtration of the KRP Buffer Extract**—In this experiment, an aliquot of KRP buffer extract was filtered through a Sepharose 6B column as described under "Materials and Methods." [3H]ADP binding activity eluted as a discrete single peak with a $K_v$ of 0.37 (Figs. 7 and 8). When the $K_v$ of the [3H]ADP binding protein was compared with that of the standards, its Stokes radius was computed to be $35 \times 10^{-8}$ cm$^3$ (Fig. 8). Using this molecular radius, the sedimentation coefficient determined earlier, and an estimated partial specific volume of 0.735 g/cm$^3$, an approximate molecular weight of 61,000 was calculated for the [3H]ADP binding protein. In addition, the protein had an axial ratio ($I/f_0$) of 1.09.

**DISCUSSION**

Previous studies have demonstrated ADP binding to platelets and their isolated membranes (7-11). The current studies demonstrate that ADP binding sites on the platelet membrane can be readily solubilized by a freeze-thaw cycle followed by extraction with buffered isotonic salt solutions. These solubilized binding sites could be saturated with [3H]ADP and had the same affinity for ADP ($K_d = 3.8 \times 10^{-7}$ M) as the membrane-bound material ($K_d = 1.5 \times 10^{-7}$ M) (11). The binding of [3H]ADP to the solubilized material was reversible, temperature-dependent, enhanced by divalent cations, and inhibited by modification of sulfhydryl groups or proteolytic digestion. All these properties of the soluble binding material are similar to those previously reported for the membrane-associated binding activity.

Both ADP and ATP complete equally with [3H]ADP for the soluble binding protein. Higher concentrations of AMP were needed to inhibit [3H]ADP binding, while adenosine had no effect. These studies imply that the ability of nucleotides to compete with [3H]ADP is related in part to the number of phosphate groups attached to the adenosine base and fit with the observation that ATP and AMP can competitively inhibit ADP-induced aggregation (19). The fact that adenosine inhibits aggregation but does not compete for [3H]ADP binding is not inconsistent, since adenosine appears to exert its major with the protein will cause $I$ to deviate from this average value and invalidate the subsequent calculations. Neither lipid nor carbohydrate binding could be estimated directly. However, since the protein was readily extracted into aqueous buffer substantial lipid binding seemed unlikely.
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inhibitory effect by stimulating platelet adenylate cyclase activity (20). This property of adenosine is probably exerted by binding to another membrane site which has been demonstrated in a variety of cells in addition to platelets (21).

There are two differences between the results presented here on the solubilized [3H]ADP binding protein and those reported previously by Nachman and Ferris (11) who studied membrane-bound material. First, the current studies indicate that significant nucleoside diphosphate kinase activity is present in the membrane and is solubilized along with the ADP-binding activity. This is consistent with numerous reports which state that nucleoside diphosphate kinase activity is loosely associated with whole platelets (2). In contrast to these observations, Nachman and Ferris concluded that their platelet membrane preparation lacked any nucleoside diphosphate kinase activity. Most likely, this discrepancy is due to their failure to include an exogenous high energy phosphate donor in their assay for nucleoside diphosphate kinase. It could also relate to different modes of membrane preparation.

A second difference between the present studies on the soluble protein and the earlier reports by Nachman and Ferris is the observation that ATP can compete as effectively as ADP for [3H]ADP binding. This was true even if the ATP was purified by ion exchange chromatography immediately before assay to eliminate any traces of ADP. Nachman and Ferris used unpurified ATP and a similar experimental protocol report that ATP was somewhat less effective than ADP is competing for the membrane-bound site (11). The reason for this discrepancy is not clear although there are other reports, like ours, that demonstrate close competition between ADP and ATP for binding to platelet membranes (8, 9).

The ADP-binding protein has many of the properties of a receptor for ADP. However, one unusual feature is that its interaction with the platelet membrane is unlike the association usually described for other receptors. Most of the receptor proteins which have been studied to date are firmly embedded in the lipid bilayer of the plasma membrane and are only solubilized with detergent containing solutions (22-24). In contrast, the ADP-binding protein can be solubilized with low ionic strength salt solutions. Although the solubilization of membrane receptors by isotonic solutions is unusual, there have been other reports of saline-soluble membrane proteins. Spectrin, a protein that accounts for nearly 35% of the red cell membrane mass, is extracted and solubilized with slightly basic solutions of low ionic strength (25). Despite the loose association implied by its easy extraction, spectrin appears to play a major role in maintaining red cell shape. Furthermore, studies by Clarke have demonstrated that 50 to 60% of mitochondrial membrane proteins can be solubilized by freezing and thawing mitochondria in isotonic solutions (26).

Recently, Jamieson and co-workers described a platelet glycoprotein, glycocalycin, which is cleaved during platelet membrane isolation to liberate a soluble macroglycopeptide (27, 28). This suggests that the ease with which the ADP-binding protein is dissociated from the platelet membrane does not accurately reflect its normal association with the membrane in the intact platelet, but reflects an artifact induced by platelet lysis and membrane purification. The relatively spherical shape of the binding protein (f/f0 = 1.09) is unusual for an intrinsic protein and also suggests that it may actually be anchored to the platelet membrane by a large hydrophobic polypeptide tail, which is cleaved from the body of the protein during membrane isolation.

While this scheme has not been demonstrated for the ADP-binding protein, it has been demonstrated that the hydrophobic portion of cytochrome b5 and cytochrome b6 reductase, which is normally embedded in the lipid bilayer of the mitochondrial membrane, are readily cleaved from the heme portion of the molecule (29).

The ADP-binding protein sedimented as a diffuse zone in the high density region of a sucrose gradient which contained 0.05 M KCl. When the KCl concentration was increased to 0.6 M, the binding activity was localized in a more discrete zone of the gradient. This change in hydrodynamic properties suggests a dissociation of the binding protein from other proteins in the impure protein mixture which is induced by 0.6 M KCl.
Radiolabeled 5'-fluorosulfonyl benzoyl adenosine, a synthetic ADP analogue, can be used as an affinity label and is covalently linked to a polypeptide of M, = 50,000 following incubation with intact platelets (30). This binding reaction was also inhibited by ADP and ATP but not by adenosine. This similarity in molecular weight and binding properties suggests that the two techniques may be identifying the same membrane protein.

Our studies employing solubilized membrane proteins provide strong evidence that ADP binding per se can be dissociated from enzymatic transformation. While the saline-soluble fraction of platelet membranes described here contained nucleoside diphosphate kinase activity, this enzymatic activity could clearly be separated from ADP binding activity by sucrose density ultracentrifugation. Furthermore, purified beef liver nucleoside diphosphate kinase did not bind [3H]ADP under the conditions employed for binding to the platelet fraction. These results imply that the ADP binding protein is distinct from platelet nucleoside diphosphate kinase.

As mentioned previously, a Mg2++, Ca2+-dependent ecto-ATPase activity has been implicated in several models of ADP-induced aggregation. Furthermore, some investigators have suggested that this ecto-ATPase activity may be attributed to platelet surface actomyosin. The apparent presence of actomyosin on the platelet surface is still disputed (31). In a recent study using highly purified antibody to human platelet myosin we were unable to show any impairment of platelet function or any surface binding of antibody (32) suggesting that little or no actomyosin is accessible to the platelet surface. While the results presented in the current study do not preclude the possibility that the ADP-binding protein has intrinsic ATPase activity, the size of the protein is not comparable to the known molecular weight of actomyosin or its component parts, G-actin (45,000) and myosin (200,000). Furthermore, the solubility of the ADP-binding protein in 0.05 M KCl is unlike either G-actin or myosin. This suggests that the ADP-binding protein is probably distinct from these platelet contractile proteins.

Although the molecular mechanism for ADP-induced platelet aggregation is still not fully characterized, like other forms of aggregation, it probably involves a complex series of biochemical events. Exposure to aggregating agents produces changes in adenylate cyclase activity, production of prostaglandin endoperoxides and thromboxane A2, and to transient changes in the levels of intracellular calcium (33). Initiation of these biochemical signals involves an interaction with specific membrane receptors. In the studies reported here, we have described the properties of M, = 60,000 protein solubilized from platelet membranes which binds ADP with a high affinity and is physiochemically distinct from membrane enzymes that metabolize ADP. It seems likely that this protein functions as an ADP receptor and that the binding of ADP to this protein is responsible for initiating platelet aggregation.

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Solubilization and characterization of a platelet membrane ADP-binding protein.

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