Identification of a Nucleotide-binding Site on Glycoprotein IIb

RELATIONSHIP TO ADP-INDUCED PLATELET ACTIVATION*

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Formalin-fixed platelets have been used to study the binding of adenine nucleotides in order to avoid the complications of nucleotide metabolism and to achieve steady-state binding. S₄-adenosine-5'-[1-thiotriphosphate] (S₄-ATP-a-S) binds to platelets at two sites (Kd₁ 3 nM; 31,000 sites/platelet; Kd₂ 200 nM; 300,000 sites/platelet) as compared with values for ADP under these conditions (Kₐ 30 nM; 25,000 sites/platelet and Kd₂ 3 μM; 400,000 sites/platelet) (bound/total ~0.1). Competition binding experiments showed that both of the ATP-α-S sites were accessible to ADP and vice versa. [³⁵S]ATP-α-S was photoaffinity cross-linked to unfixed platelets by direct irradiation with ultraviolet light. A single radiolabeled component (120 kDa) was identified and shown to be identical with the alpha subunit of GPIIb based on two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting with anti-GPIIb monoclonal antibodies, by isoelectric focusing (pI 4.5–5.5), by immunoaffinity adsorption using monoclonal anti-GPIIb/IIIa antibodies coupled to Sepharose, and by crossed immunoelectrophoresis. Amino-terminal sequencing of a tryptic fragment labeled with [³⁵S]ATP-α-S identified an 18-kDa domain beginning at Tyr-198 in the primary sequence of GPIIb. These studies demonstrated the presence of an adenine nucleotide-binding site on GPIIb.

Despite the importance of ADP in platelet function, the mechanisms by which it activates platelets are imperfectly understood. For example, it has not been resolved whether platelet activation is mediated by a single type of receptor that is coupled to two pathways, one inducing activation and the other affecting adenylyl cyclase, or whether there are two different receptors each separately affecting one of the pathways. The first, or single receptor hypothesis, is supported by the constant ratio between the activities of a wide range of structurally diverse ADP analogues in their effects as agonists or antagonists of platelet activation and the inhibition of cAMP accumulation (Cusack and Hourani, 1982a, 1982b). The two-receptor hypothesis is supported by the fact that the adenosine analogue 5'-fluorosylbenzoyl adenosine inhibits ADP-induced platelet aggregation but does not affect cAMP accumulation (Mills et al., 1985) while, conversely, p-chloromercuribenzenzene sulfonate inhibits the effect of ADP on cAMP accumulation but does not inhibit aggregation (Mills and MacFarlane, 1977).

A major difficulty in obtaining kinetic binding data on the interaction of ADP with platelets has been their ability to rapidly metabolize the ligand and its analogues and the complicating effects of ADP secretion from the activated platelets. We have recently developed the use of fixed platelets to avoid these difficulties and to provide an indicator system for measuring the ability of ADP analogues to compete with ADP under steady-state conditions (Jefferson et al., 1988; Agrawal et al., 1989). Of 20 ADP analogues examined, the most effective competitor at the high affinity site was the S₄ diastereoisomer of adenosine 5'-O-(1-thiotriphosphate) (ATP-α-S) which bound about 10 times more strongly than did ADP itself (Agrawal et al., 1989). It has been clearly established that essentially any modification in the structure of ADP, whether in the purine, ribose, or phosphate moieties, results in the loss of platelet aggregating activity. The only exception to the strict structural requirements for ADP analogues in inducing aggregation is if substitution occurs in the C-2 position (for review, see Haslam and Cusack, 1981). Attempts to identify the platelet ADP receptor by affinity labeling techniques have been constrained by this structural limitation. 2-Azidoadenosine-5'-diphosphate labeled several components in intact platelets but none were competed by ADP suggesting that nonspecific labeling may have occurred (MacFarlane et al., 1982). We have synthesized 2-aminopropylthio-ADP but found it only about one-seventh as effective an agonist as ADP (Jefferson et al., 1987) and have subsequently found that its even larger [³⁴]arylazide analogue was impractical as a photoaffinity probe due to low incorporation and low specific radioactivity.¹ The adenosine analogue 5'-fluorosylbenzoyl adenosine, which has been used to identify adenosine nucleotide-binding sites in over 40 isolated proteins (Colman, 1983), has been shown to label a 100-kDa membrane protein in intact platelets (Bennett et al., 1978), but the role of this protein in ADP-induced activation has not been clearly established (Figures et al., 1987; Colman, 1988).

Photoaffinity labeling of a specific site on a molecule usually requires a ligand substituted with a specific photolabile group (Chowdry and Westheimer, 1979). However, in a number of cases heterocyclic aromatic compounds have been photoactivated and directly coupled to cellular substrates by ultraviolet irradiation in the absence of photolabile substituents. This was first achieved in binding cyclic AMP to receptors in testicular homogenates (Antonoff and Ferguson, ¹ N. J. Greco, N. N. Tandon, and G. A. Jamieson, unpublished results.

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were determined to be, respectively, ~30 and 15% in the centrifugation assay technique and approximately 4 and 75% by the filtration assay technique.

Photoaffinity Labeling—Washed platelets (2 × 10^9) in modified Tyrode buffer B (with 1 μg PGE/ml but without bovine serum albumin) were mixed with \[^{35}S\]ATP-α-S at a concentration of 85 nm, corresponding to 30 times the Kd of the high affinity binding site as determined on fixed platelets, in a total volume of 100 μl. Samples were immediately photolyzed on ice at 254 nm on white ceramic plates at a distance of 4 cm with a UVS-54 Mineralight lamp (UltraViolet Products, San Gabriel, CA) using a Woods-type filter. Optimal irradiation time was determined to be 5 min. A 4.5-mm thick quartz cuvette filled with buffer B was placed between the incubation well and the ultraviolet lamp. Incident energy was 360 microwatts/cm² as determined by a UV meter (model 2325, UltraViolet Products).

To determine the ability of nucleotides to compete with \[^{35}S\]ATP-α-S in photoaffinity labeling requires special care to ensure that the same amount of incident energy is available both in the primary well and in the well containing the high concentration of competing nucleotide. To ensure this, experiments were carried out in parallel: in one case the quartz cuvette placed between the source and the reaction well contained buffer B while the reaction well contained \[^{35}S\]ATP-α-S plus the appropriate concentration of the competing nucleotide. In the other case, the quartz cuvette contained the same concentration of competing nucleotide in buffer B while the well contained \[^{35}S\]ATP-α-S alone. This experimental configuration ensured that any observed decrement in binding was due to the presence of the unlabeled nucleotide and not to the absorption of ultraviolet radiation by the competing nucleotide. After photolysis, free ligand was removed by centrifugation (12,000 g, 2 min) of the platelets through 10% sucrose in buffer B containing PGE. The platelet pellet was washed without resuspension with the same buffer to remove unincorporated \[^{35}S\]ATP-α-S and the pellet was then solubilized for 15–20 h with solubilization buffer (1% Triton X-100 in 50 mM Tris, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 50 μg of leupeptin/ml at pH 7.4). Triton X-100-soluble material was recovered by ultracentrifugation for 30 min at 210,000 g.

Immunoprecipitation—Purified AP-2 (1.86 mg) was coupled to 0.4 g of cyanogen bromide-activated Sepharose 4B (93% cross-linking) as per manufacturer's instructions (Pharmacia LKB Biotechnology Inc.). AP-2 immunoprecipitate fractions, stored in 50 mM Tris, 0.2 M NaCl, 0.02% NaN₃, pH 7.2, or in phosphate-buffered saline, pH 7.2, containing 0.02% NaN₃ were washed with phosphate-buffered saline and equilibrated in solubilization buffer containing 5 mM Ca²⁺. Typically 1 × 10^10 platelets labeled with \[^{35}S\]ATP-α-S were solubilized in Triton X-100 and then treated with 1 mM Ca²⁺ to ensure formation of the GPIIb/IIIa complex (Kunkel et al., 1981, 1989). The labeled complex (0.165 μg) was then incubated with 0.5 M NaCl, 0.1% Triton X-100 followed by five column volumes of solubilization buffer containing 1 mM Ca²⁺ and 0.1% Triton X-100. GPIIIb/IIIa was then eluted with 0.1 M glycine, 0.1 M NaCl, 0.1% Triton X-100, pH 2.8; the eluted fractions were immediately neutralized by the addition of 0.025 volume of 1 M Tris.

Isolation and Enzymatic Digestion of GPIIb—For sequence analysis, \[^{35}S\]ATP-α-S-labeled platelets from 25 irradiated wells (1 × 10^9 platelets) were pooled and solubilized, and labeled GPIIb/IIIa was isolated as described above. The labeled fraction eluted from AP-2-Sepharose was then concentrated on a Centricon 30 microconcentrator (Amicon, Danvers, MA). The concentrate (50 μl) was diluted with 10 volumes of phosphate-buffered saline using a Centricron 30 microconcentrator (Amicon, Danvers, MA). The concentrate (50 μl) was diluted with 10 volumes of phosphate-buffered saline, pH 7.2, containing 0.1% Triton X-100 and reconstituted before proteolysis. Digestion of \[^{35}S\]ATP-α-S-labeled GPIIIb/IIIa was carried out using TPCK-treated trypsin in phosphate-buffered saline containing 0.1% SDS and 100 μM dithiothreitol for 2 h at 23°C with a 1:20 (w/v) enzyme to substrate ratio (D’Souza et al., 1990). To inactivate the enzyme after digestion, samples were heated at 70°C for 10 min after the addition of one-fourth volume of sample buffer containing 5% (v/v) β-mercaptoethanol, 0.1 M Tris-HCl, 20 mM EDTA, 10% glycerol, 4% acrylamide stacking gel then into a 8 M urea, 12.5% acrylamide gel (13 x 18 cm) to optimize separation between oligopeptides (Swank and Munckes, 1971), electrophoretically transferred (50 mA) to Im-
mobilon (Millipore Corp.), subjected to autoradiography to detect radioactive fragments of $[^{35}S]ATP-\alpha-S$-labeled GPIIb which were then excised from Immobilon and examined by gas-phase sequencing for NH$_2$-terminal analysis (Moos et al., 1988; Tempest and Riviere, 1989) using an Applied Biosystems 477A pulsed liquid-phase sequencer and on-line model 120A phenylthiohydantoin analyzer. The initial yield for the 18-kDa fragment was 11.2 pmol.

RESULTS

Binding Studies—In our previous studies with the fixed platelet system, we used centrifugation through silicone oil (Jefferson et al., 1988) or direct centrifugation (Agrawal et al., 1989) to separate platelets with their bound, radiolabeled nucleotide from unbound ligand but B/T ratios were 0.2–0.3. Since, in the present work, we have maintained a B/T ratio ~0.1 we have repeated our studies on the binding of $[^{3}H]ADP$ to platelets and on the ability of various nucleotides to compete in this binding using both direct centrifugation and a semiautomated filtration system to separate bound from unbound ligands. Competition binding isotherms are shown in Fig. 1. Under both sets of conditions, essentially identical binding parameters were obtained corresponding to two-site models (Table I): ADP bound to high ($K_d$, 30 nm) and low ($K_d$, ~3 $\mu$m) affinity sites while ATP-\alpha-S bound with about 10-fold greater affinity to both high ($K_d$, 3 nm) and low ($K_d$, ~0.2 $\mu$m) affinity sites. There was also good agreement between the two techniques in $K_d$ values obtained from heterologous competition binding studies measuring the ability of ATP-\alpha-S to compete with ADP and vice versa.

There was also good agreement in the total number of high affinity binding sites/platelet (~25,000) from both the heterologous and homologous competition binding studies using both the centrifugation and filtration techniques. There was, however, considerable variation seen in the number of low affinity sites/platelet as reflected both in the standard errors within one series of experiments and the different values obtained in different experiments. These differences arise from wide biological variations in the number of low affinity sites between different donors.

Several experiments were carried out to determine whether the various manipulations associated with the photoactivation experiments (for example, exposure to 4 $^\circ$C and irradiation at 254 nm) altered the binding of $[^{3}H]ADP$ to platelets. These experiments were done by subjecting platelets to these manipulations and then fixing them before carrying out binding assays. No significant differences were seen in binding parameters using platelets that had been processed in the presence or absence of PGE$_1$ prior to fixation, platelets that had been activated with 1 nm human $\alpha$-thrombin for 5 min at 37 $^\circ$C without stirring in the absence of PGE$_1$, or using platelets that had been irradiated under conditions of the photoaffinity labeling experiments prior to or after fixation (data not shown).

Inhibition of Activation—We have confirmed (Cusack and Hourani, 1982a) the inhibitory effects of ATP-\alpha-S on ADP-induced platelet aggregation with an approximate EC$_{50}$ of 2 $\mu$m (Fig. 2A). In the absence of added fibrinogen, ADP induced only shape change which could also be inhibited in a dose-dependent fashion by ATP-\alpha-S (Fig. 2B). In the presence of 50 nm PGI$_2$, basal cAMP levels increased from 3.1 ± 0.6 pmol/2 × 10$^6$ platelets to 97 ± 26 pmol/2 × 10$^6$ platelets (mean ± S.D., $n$ = 3 assayed in duplicate or triplicate). ADP (2.5 $\mu$m) reduced the PGI$_2$-stimulated cAMP levels by 77 ± 4% to 24 ± 18 pmol/2 × 10$^6$ platelets. When ADP and ATP-\alpha-S were added simultaneously to PGI$_2$-stimulated platelet suspensions, only 54 ± 17% and 18 ± 6% reductions in stimulated cAMP levels were observed using 2.5 and 5.0 $\mu$m ATP-\alpha-S, respectively, demonstrating that ATP-\alpha-S antagonized the ADP-dependent inhibition of the PGI$_2$-stimulated elevation of cAMP.

Photoaffinity Labeling—By using isolated platelet membranes (Barber and Jamieson, 1970, Harmon et al., 1991), $[^{35}S]ATP-\alpha-S$ photolabeled three components of 120, 108, and 39 kDa together with several additional minor bands (data not shown). In contrast, photoaffinity labeling of intact platelets with $[^{35}S]ATP-\alpha-S$ yielded a single band of radioactivity which had a molecular mass of 120 kDa (red),135 kDa (unred) (Fig. 3, lanes 1 and 3). Photoincorporation of $[^{35}S]ATP-\alpha-S$ into this component was completely blocked in the presence of 850 $\mu$m ADP, ATP, or ATP-\alpha-S (Fig. 3, lane 2) taking the precautions described under “Materials and Methods” to ensure that there was equal ultraviolet illumination in both the control sample and in the sample containing the competing nucleotide. Photoincorporation was not affected by the presence of PGE$_1$ (1 $\mu$g/ml), Ca$^{2+}$ (1 mm), Mg$^{2+}$ (1 mm), or 0.35% bovine serum albumin but was decreased 60% if irradiations were carried out at 22 $^\circ$C as compared with those at 4 $^\circ$C, and was reduced approximately 90% in the

![Fig. 1. Competition binding.](image-url)
Fig. 2. Effect of ATP-α-S on ADP-induced activation of gel-filtered platelets. Panel A, gel-filtered platelets (1.1 × 10^8 platelets/ml) were incubated < 5 s with ATP-α-S (curve 1, no addition; curve 2, 2.5 μM; curve 3, 5 μM) before the addition of 2.5 μM ADP. Curve 4 demonstrates aggregation with 2.5 μM ADP and 1 mM Ca^{2+} but without fibrinogen. Panel B, dose-dependent inhibition of shape change in the absence of exogenous fibrinogen (curve 1, without ATP-α-S; curve 2, 2.5 μM ATP-α-S; curve 3, 5 μM ATP-α-S).

Fig. 3. Photoaffinity labeling of intact platelets with \[^{35}S\]ATP-α-S. Platelets (2 × 10^9/ml) were irradiated at 254 nm (360 microwatts/cm^2) with \[^{35}S\]ATP-α-S (85 nM) for 5 min, solubilized, and analyzed by 7% SDSPAGE and subjected to autoradiography. Lane 1, Triton X-100-soluble fraction (reduced); lane 2, Triton X-100-soluble fraction of platelets irradiated in the presence of 850 μM ADP; lane 3, Triton X-100-soluble fraction (nonreduced); lane 4, Triton X-100-insoluble fraction (reduced). Covalent incorporation of \[^{35}S\]ATP-α-S into GPIIb was light dependent and did not occur in its absence. The results shown are representative of at least 12 similar experiments performed using different cell preparations and different batches of \[^{35}S\]ATP-α-S.

Fig. 4. Autoradiogram of two-dimensional nonreduced/reduced SDS-PAGE of \[^{35}S\]ATP-α-S-labeled intact platelets (1 × 10^9, top) or surface iodinated intact platelets (1 × 10^9, bottom) utilized as a glycoprotein reference. The dotted circle indicates the relative position of GPIIIa. All samples were derived from Triton X-100-soluble material.

Nucleotide-binding Site on GPIIb

Table I

| Binding parameters (K_D and K_I) for adenine nucleotides using direct centrifugation (C) or filtration (F) systems | Binding constants | S1 | S2 |
|---|---|---|---|
| [H]ADP versus ADP | 7 (C) | 30 ± 1 | 3 ± 1 | 25,600 ± 4,200 | 22,300 ± 4,100 | 383,000 ± 103,000 | 101,000 ± 22,000 |
| [H]ADP versus ATPαS | 8 (C) | 22 ± 6 | 0.8 ± 0.2 | 32,900 ± 4,800 | 889,000 ± 326,000 |
| [H]ATPαS versus ATPαS | 6 (F) | 3 ± 0.4 | 0.2 ± 0.04 | 23,900 ± 4,500 | 82,000 ± 15,500 |
| [H]ATPαS versus ADP | 6 (C) | 6 ± 0.5 | 0.97 ± 0.32 | 42,684 ± 8,200 | 136,000 ± 61,000 |

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Characterization of GPIIbα—The 120-kDa component was identified as GPIIbα by two-dimensional electrophoresis (Fig. 4), isoelectric focusing (pI 4.5–5.5) (Fig. 5), and crossed immunoelectrophoresis (Fig. 6). The \[^{35}S\]ATP-α-S-labeled GPIIb/IIIa complex was immunopurified on a column of AP-2-Sepharose (Fig. 7) and digested with trypsin (1:20) yielding a single radiolabeled band (~18 kDa) as well as three silver staining bands which were also found in trypsin autolysates in the absence of GPIIIb (Fig. 8). Gas-phase sequencing of tryptic peptides derived from the labeled 18-kDa peptide was performed using a matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) and a tandem mass spectrometer (MS/MS). The peptide was subjected to peptide mass fingerprinting (PMF) and peptide sequence analysis (PSA) using the SEQUEST software. The results indicated that the 18-kDa peptide was covalently incorporated into GPIIbα.

The 224 kDa band was immobilized onto a column of AP-2-Sepharose and eluted with 0.1 M NaOH. The eluate was concentrated and subjected to SDS-PAGE and Western blotting using an antibody specific to GPIIbα. The 224 kDa band was covalently incorporated into GPIIbα as confirmed by Western blotting.

The 120 kDa band was covalently incorporated into GPIIbα as confirmed by Western blotting using an antibody specific to GPIIbα. The results indicated that the 120 kDa band is covalently incorporated into GPIIbα.
The sequence for GPIIb, in human erythroleukemia (HEL) cells precipitating anti-whole platelet antibodies, was identified as GPIIb/IIa by including [³²P]ATP in the intermediate for a trypsin-generated peptide. Based on a molecular mass (Poncz et al., 1987) and is preceded by arginine as expected for amino acids 350-360 in the GPIIb protein sequence. The identified amino-terminal sequence showed no homology to sequences in GPIIIa (Fitzgerald et al., 1987), GPIIb (Poncz et al., 1987), trypsin, or platelet actin (Intelligenetics Suite, Release 5.37, 1990). In these experiments, undigested [³²P]ATP-α-S-labeled GPIIb, was included as a positive control and gave the expected NH₂-terminal sequence of LNLDP-VQLXF (Poncz et al., 1987).

**DISCUSSION**

This report demonstrates that GPIIb, contains a nucleotide-binding site which meets many of the requirements expected for a platelet ADP receptor. This binding site was recognized by direct photoaffinity labeling with [³²P]ATP-α-S, and this labeling was competable by the agonist ADP, and the antagonists ATP-α-S and ATP but not by adenosine which does not antagonize ADP-induced platelet activation. This pattern of competition is consistent with our binding studies using the fixed platelet system. ATP-α-S competed at all sites accessible to ADP and, conversely, ADP competed at all sites accessible to ATP-α-S. This implies that the photolabeling of GPIIb, seen with [³²P]ATP-α-S represents a binding site that is also completely accessible to ADP and that sites that are not accessible to ADP would not photolabel with [³²P]ATP-α-S. Our failure to detect similar photolabeling in experiments using [³⁴S]ATP and [³⁴C]ADP probably reflects poor incorporation due to the fact that its binding affinity (Kd 30 nM) is much weaker than that of ATP-α-S (Kd 3 nM), that it is rapidly metabolized to even more weakly bound products and that the available labeled forms of ADP are of much lower specific radioactivity than that available for [³²P]ATP-α-S.

The 18-kDa ATP-α-S-binding peptide isolated from human GPIIb, has the amino-terminal sequence YAEAGFSSVVT (K/Q)AGEL beginning at Tyr-198. A corresponding sequence of 18 kDa the peptide would be expected to extend to one of the several Lys or Arg residues occurring in the region of the homologous 16-mer YAEAGFSSVVT(K/Q)AGEL. This sequence is homologous to the sequence YCEAGFSSVVTQA- GEL beginning at Tyr-198 as determined from the cDNA sequence for GPIIb, in human erythroleukemia (HEL) cells (Poncz et al., 1987) and is preceded by arginine as expected for a trypsin-generated peptide. Based on a molecular mass the sequence for GPIIib, in human erythroleukemia (HEL) cells precipitating anti-whole platelet antibodies.

**FIG. 5. Autoradiogram of two-dimensional isoelectric focusing/SDS-PAGE slab gel of intact platelets (1 × 10⁶; Triton X-100-soluble fraction) irradiated with [³²P]ATP-α-S. First dimension, isoelectric focusing; pH 3.5-10; second dimension, 7% SDS-PAGE. The arrow indicates GPIIb; note the absence of radio-labeled GPIIb₁α.**

**FIG. 6. Crossed-immunoelectrophoresis.** The Triton X-100-soluble fraction was electrophoresed in the second dimension against precipitating anti-whole platelet antibodies. Panel A, staining with Coomassie Blue. Panel B, autoradiography of platelets radiolabeled with [³²P]ATP-α-S. The labeled peak (panel B) was conclusively identified as GPIIb/IIa by including [³²P]-AP-2 in the intermediate gel (data not shown).

**FIG. 7. Immunopurification on AP-2-Sepharose.** Intact platelets, irradiated in the presence of [³²P]ATP-α-S, were solubilized with 1% Triton X-100, and the soluble material was applied to an AP-2-Sepharose column. Fractions were analyzed by 7% SDS-PAGE. A silver-stained gel is shown in panel A and the corresponding autoradiogram in panel B. Lane 1, Triton X-100-soluble fraction; lanes 2 and 3, nonadsorbed material; Elution fractions, obtained by washing the column with glycine-HCl.

**FIG. 8. Tryptic digest of [³²P]ATP-α-S-labeled GPIIb/IIa.** GPIIb/IIa isolated from [³²P]ATP-α-S-labeled platelets was proteolyzed for 2 h at 22 °C with a 20:1 (w/w) ratio of labeled GPIIb/IIa to TPKC-treated trypsin. Proteolysis mixtures were separated by 8 M urea, 12.5% acrylamide gel, transferred to Immobilon, then subjected to autoradiography. The radiolabeled band was excised and sequenced directly. Panel A, silver-stained gel. Panel B, autoradiography of Immobilon strip. The open arrows indicate silver-stained components derived from trypsin in the absence of GPIIb/IIa.
can be identified in the cDNA-deduced amino acid sequence from HEL cells (YCEAGFSSVTVQAGEL; Poncz et al., 1987; Heidenreich et al., 1990) which differs, however, from the platelet sequence in having Cys rather than Ala at position 199. In addition, we have consistently found an equimolar mixture of Lys and Gln at position 209 rather than Gln alone, as in the HEL cell clone: this may represent a polymorphic variation in platelet GPIIb, at this position. These variations between the primary (platelet) and deduced (HEL) amino acid sequences are unlikely to be due to a related integrin α-subunit which complexes with GPIIb/IIIa in view of the fact that the NH2-terminal sequence for GPIIb, determined in control experiments is identical with that deduced from HEL cells.

The present steady-state binding studies were carried out under conditions such that the ratio (B/T) of bound/total ligand was ~0.1 at the lowest concentration of ligand examined (Bylund and Yamamura, 1990). Under these conditions the affinity constants for ADP were ~5–10-fold less than those previously determined at higher B/T ratios, while the number of high affinity sites was correspondingly fewer (Jefferson et al., 1988; Agrawal et al., 1989). These conclusions were confirmed by redetermining the binding parameters under the original conditions of B/T = 0.2–0.3 (data not shown). The number of high affinity sites calculated for the steady-state binding of ADP and ATP-α-S in the present studies range from 18,500 ± 2,500 to 32,000 ± 4,800 and agree well with the number of GPIIb/IIIa complexes from the binding of fibrinogen to stimulated platelets (14,000–38,000 sites/platelets) (Marguerie et al., 1980; Peerschke and Zucker, 1981; Plow and Marguerie, 1982; Plow et al., 1985) although higher numbers of binding sites have been reported for the binding of monoclonal antibodies ranging from about 40,000/platelet for antibodies against GPIIb (McEver et al., 1980; Gulino et al., 1990) to about 50,000/platelet for antibodies against the GPIIb/GPIIIa complex (Kunicki et al., 1981; Bennett et al., 1982; McEver et al., 1983).

The components labeled with [35S]ATP-α-S using isolated membranes were not extensively examined because their multiplicity indicated a much lower specificity of labeling than that seen with intact platelets. On the basis of their electrophoretic mobility the bands of 120 and 108 kDa were taken to be GPIIb and GPIIIa, respectively (Poncz et al., 1987; D'Souza et al., 1990). We have confirmed earlier observations (Cusack and Hourani, 1982b) that ATP-α-S antagonizes ADP-induced platelet aggregation and have shown that it decreases ADP-induced inhibition of PGL-stimulated adenylyl cyclase. We have extended these results to show that ATP-α-S also inhibits ADP-induced shape change and serotonin release. We have also demonstrated that ATP-α-S competes in the binding of ADP to platelets and that it photolabels a specific domain of GPIIb, in intact platelets.

While the present studies have identified an ADP-binding site on GPIIb, much remains to be done to determine whether this binding site functions as a receptor, occupancy of which is necessary for ADP-induced platelet activation. In particular, binding and photolabeling studies with Glnzmann's platelets, which show normal ADP-induced Ca2+ influx (Powl and Hardisty, 1985), are required as well as the localization of the binding domain to a precise amino acid sequence and the demonstration that domain-specific antibodies or peptide segments inhibit ADP-induced platelet activation.

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