Probing Chemical and Conformational Differences in the Resting and Active Conformers of Platelet Integrin αIibβ3

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Integrin αIibβ3 is the fibrinogen receptor that mediates platelet adhesion and aggregation. The ligand binding function of αIibβ3 is “activated” on the platelet surface by physiologic stimuli. Two forms of αIibβ3 can be purified from platelet lysates. These forms are facsimiles of the resting (Activation State-1 or AS-1) and the active (Activation State-2 or AS-2) conformations of the integrin found on the platelet surface. Here, the differences between purified AS-1 and AS-2 were examined to gain insight into the mechanism of activation. Four major findings are put forth. 1) The association rate (kapp) between purified AS-1 and AS-2 were examined to verify that integrin is unrelated to activation. 3) Peptide mass fingerprints indicate that the chemical structure of AS-1 and AS-2 are virtually identical, calling into question the idea that post-translational modifications are necessary for activation. 4) The two forms of αIibβ3 have significant conformational differences at three positions. These include the junction of the heavy and light chain with the ability to bind soluble fibrinogen (Fg) present in the plasma. Because Fg is a dimer, its binding leads to platelet aggregation and ultimately halts the loss of blood. Because the concentrations of both Fg and platelets are so high in the blood, αIibβ3 must be maintained in a resting, or inactive state, to ensure proper blood flow. Therefore, an understanding of how αIibβ3 is activated is of great importance.

The precise mechanism by which the ligand binding affinity of αIibβ3, and other integrins, is modulated by cellular stimuli is still not completely understood. Several pathways to activation have been put forth. Many of these have focused on the role of the integrin cytoplasmic domains in activation (10). One hypothesis suggests that changes in the conformation of the cytoplasmic tails can release a conformational constraint or open an “integrin hinge” (11). This hinge could potentially be opened by proteolysis of the integrin cytoplasmic domains (12–14). Alternatively, the hinge could be released by phosphorylation of the integrin. The cytoplasmic tail of β3 contains several potential phosphorylation sites (15, 16). One of these phosphorylation sites, an NPXY motif within the tail of β3 is essential for integrin activation (17, 18). Therefore, phosphorylation must also be considered as a potential route to activation. Another pathway to activation may involve proteolysis within the ectodomain of the integrin. In fact, there is evidence that αIibβ3 can be activated in this manner (19). Still other work indicates that divalent ions, which bind to the integrin and control ligand affinity, may activate the integrin (20–22). The physical association of integrins with regulatory proteins (23–25) could also enact conformational changes that lead to activation. Intracellular signaling pathways like those controlled by Ha- and R-Ras (26, 27) have also been implicated in the activation of integrins.

Despite this progress, there is still no unifying hypothesis on exactly what biochemical changes occur within an integrin during activation. We reasoned that one way to gain insight into this issue was to compare the differences in the structure and function of the resting and active integrin. We have used Surface Plasmon Resonance (SPR) to compare the kinetic behavior of the active and resting form of αIibβ3 and mass spectrometry to probe the structural differences between the two conformers. The study reveals that the active and resting integrin are virtually identical in chemical structure but reveals important conformational distinctions. These observations lay the fundamental groundwork required to properly interpret other studies aimed at deciphering how inside-out signaling alters the ligand binding function of integrins.

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‡ The abbreviations used are: Fg, fibrinogen; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TOF, time-of-flight.

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Purification of αIIbβ3—The two forms of integrin αIIbβ3, AS-1 and AS-2, were purified according to previously published procedures (28–30) with minor modifications. Briefly, washed, outdated platelets were lysed in a 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 1 mM CaCl2, and 10−5 M leupeptin. The lysate was centrifuged at 50,000 × g and stored at −80 °C until further use. The lysate was rapidly thawed at 37 °C, centrifuged at 50,000 × g, and passed over Con-A-Sepharose (Amersham Pharmacia Biotech). Proteins were eluted with Buffer A (20 mM Tris buffer, pH 7.0, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.1% Triton X-100, and 0.05% NaN3) containing 200 mM methyl α-D-mannopyranoside (Sigma) and 10−5 M leupeptin (Sigma). Fractions containing αIIbβ3 were pooled and then depleted of the active form of the integrin (AS-2) by circulating over a column of KYGRGDS-Sepharose. To ensure that the flow through was depleted of AS-1, the lysate was passed over two RGD affinity columns in series. The AS-2 form of the integrin was eluted from these columns using soluble RGD peptide. AS-2 was concentrated, dialyzed against Buffer A, and stored at −80 °C. The purity and integrity of each form of the integrin was assessed by SDS-PAGE.

Peptides, Ligands, and Antibodies—Human fibrinogen was purchased from Enzyme Research Laboratories. Fab-9, a recombinant antibody containing the RGD motif (31, 32), was purified by affinity chromatography on a column of goat anti-human IgG-Sepharose. Synthetic peptides were purchased from Coast Scientific and from Anaspec. Antibodies against the C-terminal domain of αIIb, including PM1–1, anti-V41 (33) which are specific for C terminus of the heavy chain of αIIb, and the N terminus of the light chain of αIIa, respectively, were generous gifts from Dr. Mark Ginsberg (Scripps Research Institute). Cells expressing recombinant variants of Fg lacking the RGD motifs were generously provided by Dr. David Farell (Milton Hershey Medical Center, Penn State University).

Construction of Ligand Affinity Resins—For affinity purification of αIIbβ3, the peptide KYGRGDS was coupled to CNBr-Sepharose (Amer sham Pharmacia Biotech) according to the manufacturer’s specifications. To analyze the dephosphorylation status of the integrins, synthetic peptides were purchased from Coast Scientific and from Anaspec. Antibodies against the C-terminal domain of αIIb, including PM1–1, anti-V41 (33) which are specific for C terminus of the heavy chain of αIIb, and the N terminus of the light chain of αIIa, respectively, were generous gifts from Dr. Mark Ginsberg (Scripps Research Institute). Cells expressing recombinant variants of Fg lacking the RGD motifs were generously provided by Dr. David Farell (Milton Hershey Medical Center, Penn State University)

Fig. 1. Comparing the depth of the RGD binding pocket on purified AS-1 and AS-2. The resting and active conformers of αIIbβ3 were purified from outdated platelet lysates as described under “Materials and Methods.” The affinity of AS-1 (lane 1) and AS-2 (lane 2) was assessed by Coomassie staining of 7% polyacrylamide gels run under non-reducing conditions. The affinity of AS-1 and AS-2 (lanes 1 and 3) or to KYGRGDS-hexamidine-Sepharose (lanes 2 and 4). Integrin (20 μg) was mixed with 20 μl of settled affinity resin and incubated for 18 h. The affinity resins were washed extensively to remove unbound integrin. Bound integrin was removed from the resin by incubation in SDS sample buffer. The eluted integrin was analyzed on SDS-PAGE. This experiment is representative of three repetitions, making use of three different batches of purified integrin in which identical results were obtained.

Fig. 2. Binding of AS-1 and AS-2 to fibrinogen. The binding of AS-1 and AS-2 to Fg-linked plasmon resonance sensor chips was measured with the BIAcore 3000. Binding was performed in buffer containing 1 mM Ca2+ and 1 mM Mg2+. From a series of such sensorgrams in which the amount of integrin included in the analyte was varied, the association rate constant (k1) between AS-2 and Fg was derived as described (35, 36).

Fig. 3. Effect of Mn2+ on the binding of AS-1 and AS-2 to fibrinogen. To determine whether Mn2+ could convert AS-1 to AS-2, the AS-1 form of the integrin was dialyzed into buffer containing 200 μM Mn2+. A sample of this material was then dialyzed back into buffer containing 1 mM Ca2+. Then, the binding of AS-2 (●), AS-1 (□), AS-1 dialyzed into Mn2+ (■), and AS-1 dialyzed into Mn2+ and then back into Ca2+ (▲) to fibrinogen was measured with SPR. The resulting sensorgrams are shown. This experiment is one of three repetitions, making use of two separate batches of purified AS-1 and AS-2, each of which yielded nearly identical results.
Detection of tryptic peptides from AS-1 and AS-2

Integrin α\textsubscript{IIb}β\textsubscript{3} was separated on SDS-PAGE, and the heavy chain of α\textsubscript{IIb} (section A), the light chain of α\textsubscript{IIb} (section B) or the β\textsubscript{3} subunit (section C) was excised and subjected to in-gel tryptic digestion as described under “Materials and Methods.” Peptides were extracted from the gel slice and analyzed by MALDI-TOF mass spectrometry. The spectra between 800 daltons and 6000 daltons was examined for peptides. The monoisotopic mass of the predicted tryptic peptides are compared to the masses of peptides observed in the MALDI profile. The peptides observed in the spectra are in the column designated as “Native α\textsubscript{IIb}β\textsubscript{3}.” Some peptides could not be detected due to their glycosylation, so α\textsubscript{IIb}β\textsubscript{3} was enzymatically deglycosylated and analyzed in the same manner (column noted as “Deglycosylated”). Peptide fragments that were not observed due to size or detection limitations are noted as ND. The change in the mass of a fragment containing an N\textsubscript{-}linked sugar chain after deglycosylation (Asn\textsubscript{33}Asp) is an increase of 1 dalton.

### Table I: Detection of tryptic peptides from AS-1 and AS-2

| Residue numbers | Predicted mass\(^a\) | Glycosylation\(^b\) | Observed mass from AS-1\(^c\) | Observed mass from AS-2\(^c\) |
|-----------------|----------------------|---------------------|-----------------------------|-----------------------------|
|                 | Native α\textsubscript{IIb}β\textsubscript{3} | Deglycosylated | Native α\textsubscript{IIb}β\textsubscript{3} | Deglycosylated |
| 1–27            | 3012.5               | N-link (P)         | 3011.4                      | 3012.9                      |
| 28–32           | 571.3                |                     | 571.4                       | 571.7                       |
| 33–41           | 881.6                |                     | 881.7                       | 881.5                       |
| 42–59           | 2033.9               |                     | 2034.2                      | 2034.7                      |
| 60–73           | 1562.8               |                     | 1563.9                      | 1563.7                      |
| 74–77           | 520.3                |                     | 520.4                       | 520.2                       |
| 78–88           | 1223.6               |                     | 1222.7                      | 1222.4                      |
| 89–90           | 246.1                |                     | 246.2                       | 246.1                       |
| 91–118          | 3136.6               |                     | 3136.8                      | 3137.2                      |
| 119–124         | 706.3                |                     | ND                          | ND                          |
| 125–139         | 1601.8               |                     | 1598.3                      | 1600.6                      |
| 140             | 175.1                |                     | ND                          | ND                          |
| 141–147         | 882.4                |                     | 882.5                       | 882.4                       |
| 148–153         | 647.4                |                     | 647.2                       | 647.1                       |
| 154–164         | 1415.6               |                     | 1415.2                      | 1415.9                      |
| 165             | 175.1                |                     | ND                          | ND                          |
| 166–276         | 12166.8              | N-link (P)         | ND                          | ND                          |
| 277–279         | 425.3                |                     | 425.6                       | 425.7                       |
| 280–281         | 288.3                |                     | 288.3                       | 288.4                       |
| 304–317         | 1586.8               |                     | 1587.2                      | 1587.5                      |
| 318–320         | 361.2                |                     | 361.6                       | 361.2                       |
| 321             | 147.1                |                     | ND                          | ND                          |
| 322–327         | 644.4                |                     | 644.2                       | 644.3                       |
| 329–335         | 1035.6               |                     | 1035.5                      | 1035.4                      |
| 356–355         | 2022.1               |                     | 2022.7                      | 2022.1                      |
| 356–368         | 1331.7               |                     | 1331.7                      | 1331.3                      |
| 369–386         | 1779.8               |                     | 1780.1                      | 1780.3                      |
| 378–400         | 1502.8               |                     | 1502.8                      | 1502.6                      |
| 401–422         | 2353.2               |                     | 2353.8                      | 2354.0                      |
| 423–449         | 2825.4               |                     | 2825.8                      | 2826.9                      |
| 450–455         | 641.4                |                     | 641.1                       | 641.9                       |
| 456–471         | 1681.9               |                     | 1682.0                      | 1682.6                      |
| 472–479         | 933.1                |                     | 933.7                       | 933.7                       |
| 480–501         | 2459.2               |                     | 2460.5                      | 2460.9                      |
| 502–512         | 1271.7               |                     | 1271.8                      | 1271.2                      |
| 513–516         | 528.3                |                     | 528.5                       | 528.8                       |
| 517–519         | 360.2                |                     | 360.8                       | 360.3                       |
| 529             | 175.2                |                     | ND                          | ND                          |
| 521–540         | 1999.1               |                     | 2001.5                      | 2001.6                      |
| 541–553         | 1570.8               |                     | 1571.9                      | 1571.6                      |
| 554–559         | 752.3                |                     | 752.1                       | 752.2                       |
| 560–561         | 262.1                |                     | 262.4                       | 262.2                       |
| 562–597         | 3777.0               | N-link (P)         | 3773.5                      | 3775.2                      |
| 598–601         | 6849.3               |                     | ND                          | ND                          |
| 602–671         | 1121.6               |                     | 1121.5                      | 1121.4                      |
| 672–677         | 775.4                |                     | 775.5                       | 775.5                       |
| 678             | 147.1                |                     | ND                          | ND                          |
| 679–683         | 648.3                | N-link (P)         | 648.3                       | 648.3                       |
| 684–694         | 1259.6               |                     | 1261.4                      | 1260.7                      |
| 695             | 147.1                |                     | ND                          | ND                          |
| 696–724         | 3073.6               |                     | 3074.5                      | 3074.9                      |
| 725–726         | 234.1                |                     | 234.0                       | 234.1                       |
| 727–734         | 888.4                |                     | 887.9                       | 888.2                       |
| 735–742         | 1023.6               |                     | 1023.6                      | 1023.6                      |
| 744–751         | 915.5                |                     | 915.6                       | 915.4                       |
| 752–768         | 1732.9               |                     | 1732.5                      | 1732.7                      |
| 769–779         | 1260.6               |                     | 1261.8                      | 1262.9                      |
| 780–836         | 6277.2               |                     | 6281.0                      | 6280.5                      |
| 837–855         | 2085.1               | O-link (P)         | 2086.1                      | 2087.4                      |
| 856             | 175.1                |                     | ND                          | ND                          |
| 857–858         | 290.1                |                     | 290.2                       | 290.4                       |
| 859             | 175.1                |                     | ND                          | ND                          |
## TABLE I—continued

### Section B: tryptic peptides derived from the light chain of αIIb

| Residue numbers | Predicted mass | Glycosylation | Observed mass from AS-1 | Observed mass from AS-2 |
|-----------------|----------------|--------------|--------------------------|--------------------------|
| 1–12            | 1440.7         |              | ND                       | ND                       |
| 13–38           | 2991.4         |              | 2992.8                   | 2993.2                   |
| 39–41           | 360.1          |              | 360.1                    | 360.1                    |
| 42–47           | 5267.8         | N-link (P)   | ND                       | ND                       |
| 88–98           | 1300.7         |              | 1300.5                   | 1300.5                   |
| 99–103          | 617.3          |              | 617.2                    | 617.8                    |
| 104–130         | 2987.8         |              | 2984.5                   | 2986.2                   |
| 131–135         | 597.2          |              | 595.6                    | 595.6                    |
| 136             | 175.1          |              | ND                       | ND                       |
| 137–138         | 289.2          |              | 289.1                    | 288.8                    |
| 139–149         | 1302.5         |              | 1302.7                   | 1302.8                   |

### Section C: tryptic peptides derived from the β3 subunit

| Residue numbers | Predicted mass | Glycosylation | Observed mass from AS-1 | Observed mass from AS-2 |
|-----------------|----------------|--------------|--------------------------|--------------------------|
| 1–8             | 918.5          |              | 918.7                    | 918.0                    |
| 9–37            | 3223.3         |              | 3223.2                   | 3223.1                   |
| 38–41           | 535.3          |              | 535.4                    | 535.5                    |
| 42–46           | 616.4          |              | 617.1                    | 617.1                    |
| 47–62           | 1820.8         |              | 1821.1                   | 1821.5                   |
| 63–72           | 1171.6         |              | 1171.9                   | 1171.8                   |
| 73–87           | 1532.7         |              | 1533.6                   | 1533.7                   |
| 88–91           | 472.3          |              | 472.4                    | 472.5                    |
| 92–98           | 830.4          |              | 830.4                    | 831.3                    |
| 99–105          | 863.5          | N-link (P)   | 862.8                    | 863.2                    |
| 106–125         | 2472.1         |              | 2471.5                   | 2471.8                   |
| 126–137         | 1389.7         |              | 1390.9                   | 1390.2                   |
| 138–143         | 719.4          |              | 719.2                    | 719.4                    |
| 144             | 147.1          |              | ND                       | ND                       |
| 145–150         | 703.4          |              | 703.5                    | 704.1                    |
| 151–181         | 3535.7         |              | ND                       | ND                       |
| 182–191         | 1217.6         |              | 1218.4                   | 1218.6                   |
| 192–202         | 1282.7         |              | 1282.6                   | 1282.7                   |
| 203–208         | 765.4          |              | 765.2                    | 765.4                    |
| 209             | 147.1          |              | ND                       | ND                       |
| 210–214         | 576.3          |              | 576.3                    | 576.3                    |
| 215–216         | 289.1          |              | 290.2                    | 288.3                    |
| 217–235         | 2053.8         |              | 2055.4                   | 2056.2                   |
| 236–239         | 531.3          |              | 531.4                    | 531.5                    |
| 240–253         | 1531.8         |              | 1532.8                   | 1532.8                   |
| 254–261         | 882.5          |              | 882.5                    | 882.4                    |
| 262–298         | 3993.8         |              | 3995.6                   | 3996.3                   |
| 299–302         | 475.3          |              | 475.7                    | 475.4                    |
| 303–350         | 5228.7         | N-link (P)   | 5226.7                   | 5226.8                   |
| 351–352         | 288.2          |              | 288.2                    | 288.2                    |
| 353–354         | 234.1          |              | 234.1                    | 234.1                    |
| 355–360         | 744.4          |              | 743.8                    | 744.6                    |
| 361–384         | 2673.3         | N-link (P)   | 2675.4                   | 2676.1                   |
| 385–390         | 695.3          |              | 695.7                    | 695.7                    |
| 391–402         | 1266.5         |              | 1267.9                   | 1267.3                   |
| 403–404         | 274.2          |              | 274.2                    | 274.2                    |
| 405–410         | 718.3          |              | 719.1                    | 718.2                    |
| 411–412         | 276.2          |              | 276.3                    | 276.3                    |
| 413–422         | 1123.7         |              | 1123.7                   | 1123.8                   |
| 423–447         | 2921.3         |              | 2922.7                   | 2923.1                   |
| 448–461         | 1644.6         | N-link (I)   | 1645.3                   | 1645.6                   |
| 462–489         | 3374.3         |              | 3374.1                   | 3374.0                   |
| 490–498         | 1060.5         |              | 1060.3                   | 1060.7                   |
| 499–515         | 2000.7         |              | 1999.7                   | 2001.5                   |
| 516–519         | 418.2          |              | 418.5                    | 418.3                    |
| 520–530         | 1510.5         |              | 1511.4                   | 1512.0                   |
| 531–532         | 310.2          |              | 310.4                    | 310.4                    |
| 533–563         | 3754.3         | N-link (P)   | 3755.8                   | 3755.4                   |
| 564–578         | 1658.7         |              | 1659.6                   | 1659.9                   |
| 579–580         | 204.1          |              | ND                       | ND                       |
| 581–600         | 2366.9         |              | 2369.0                   | 2369.1                   |
| 601–611         | 1356.5         |              | 1357.2                   | 1356.9                   |
| 612             | 147.1          |              | ND                       | ND                       |
| 613–618         | 824.3          |              | ND                       | ND                       |
| 619             | 147.1          |              | ND                       | ND                       |
Table I—continued

| Residue numbers | Predicted mass | Glycosylation | Observed mass from AS-1′ | Observed mass from AS-2′ |
|-----------------|----------------|---------------|--------------------------|--------------------------|
|                 | mass*          | Glycosylation | Native α7β3            | Deglycosylated            |
|                 |                | (P)           |                          |                          |
| 620–622         | 437.2          |               | 437.9                    | 438.2                    |
| 623–633         | 1288.6         | N-link(I)     | 1285.1                   | 1285.8                   |
| 634–656         | 498.2          |               | 498.2                    | 498.2                    |
| 637–643         | 819.4          |               | ND                       | ND                       |
| 644–646         | 389.2          |               | ND                       | ND                       |
| 647–650         | 420.2          |               | ND                       | ND                       |
| 651–658         | 970.4          |               | ND                       | 389.5                    |
| 659–666         | 1006.4         |               | 1007.1                   | 1563.9                   |
| 667–689         | 1562.8         |               | 1564.1                   | 1563.9                   |
| 690–716         | 2801.7         |               | 2802.5                   | 2801.9                   |
| 717–724         | 981.2          |               | 981.3                    | 979.8                    |
| 725             | 147.1          |               | ND                       | ND                       |
| 726–729         | 494.6          |               | 494.5                    | 494.5                    |
| 730–734         | 709.7          |               | 709.5                    | 709.2                    |
| 735–736         | 246.2          |               | 246.2                    | 246.2                    |
| 737–738         | 219.2          |               | 218.2                    | 218.3                    |
| 739–748         | 1222.3         |               | 1222.6                   | 1221.4                   |
| 748–760         | 1404.8         |               | 1404.5                   | ND                       |
| 761–762         | 171.7          |               | ND                       | ND                       |

* The predicted mass of each peptide reflects the modifications to the mass made by reduction and alkylation.
* Potential glycosylation sites were identified by the Swiss-Prot database; (P) reflects the presence of a potential glycosylation site whose usage has been substantiated by experimental evidence. (P) indicates that the peptide contains a potential glycosylation site that is predicted by consensus sequence.

All peptides present in the spectra had masses greater than 200 daltons.

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allowed to couple to the resin for 18 h, and free peptide was removed by extensive washing with 20 mM Tris buffer, pH 7.0, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, containing 0.1% Triton X-100.

Surface Plasmon Resonance—The kinetic parameters (association and dissociation rate constants, k1 and k₋1, respectively, and the affinity constant (Kₐ) between Fg and α7β3, or Fab-9 and α7β3, were measured by SPR using methods we have described previously (35, 36).

Limited digestion of α7β3 was accomplished with Asp-N protease (Roche Molecular Biochemicals, sequencing grade). Asp-N was added to purified α7β3 (1 mg/ml) of 100 µg in 100 µl of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.1% Triton X-100. Protease was added in a ratio of 1:300 (weight to weight) and incubated with integrin at 37 °C for 4 h. The reaction was stopped by chilling to 4 °C and immediately analyzing the sample on 12% SDS-PAGE.

Protein and Peptide Mass Fingerprinting—Peptide mass fingerprinting (37, 38) was used to define the composition of α7β3 and β3 and the composition of their proteolytic fragments. Proteins or peptides were separated on 12% SDS-PAGE under non-reducing conditions. Proteins were visualized by silver staining. Gel slices containing protein were excised and then reduced and alkylated by performing the following procedure three times. Gel slices were dehydrated with acetonitrile and subjected to reduction with 20 mM dithiothreitol (DTT) at 56 °C for 1 h. Proteins embedded in the gel were then alkylated with 60 mM iodoacetamide at room temperature for 30 min. Gel slices were washed with 100 mM NH₄HCO₃, again dehydrated with acetonitrile. Gel slices containing reduced and alkylated integrin were dried in a vacuum centrifuge and then rehydrated in 20 mM NH₄HCO₃ containing 20 µg/ml of trypsin (Roche Molecular Biochemicals, modified sequencing grade).

Each gel slice was incubated with enzyme in an ice-bath for 1 h, and then at 37 °C for 18 h. Gel slices were washed with 20 mM NH₄HCO₃ and then tryptic peptides were extracted with three changes of a solution of 5% formic acid, 50% acetonitrile, and 25% isopropanol. The solution containing peptides was evaporated to dryness. Dry peptide mixtures were dissolved in 0.1% trifluoroacetic acid. To detect some domains within α7 and β3 by mass spectrometry, it was necessary to deglycosylate the protein. Deglycosylation was carried out in 20 mM sodium phosphate buffer, pH 7.5 with O-glycanase, N-glycanase, and sialidase (Roche Molecular Biochemicals) at 37 °C for 40 h.

In some cases proteins were digested in solution rather than in acrylamide gel slices. Because Triton X-100 suppresses peptide detection in MALDI mass spectrometry, it was necessary to remove this detergent. This was accomplished with the following procedure. Methanol (140 µl) was added to 35 µl of integrin in a volume of 35 µl. The sample was vortexed and centrifuged at 9000 × g for 10 s. Then, chloroform (35 µl) was added to the solution and vortexed, and the sample was centrifuged again. Finally, water (90 µl) was added and the sample vigorously vortexed. Phase separation was enacted by centrifugation for 1 min. The upper phase was discarded. The lower phase, containing chloroform, and the interface, comprised of precipitated α7β3, were retained. Methanol (90 µl) was added to the remainder of the sample to precipitate the α7β3. Precipitated integrin was recovered by centrifugation for 2 min at 9,000 × g. The supernatant was discarded and the protein pellet was air dried. Then the protein was resuspended in 20 mM NH₄HCO₃ containing 0.2% octylglucoside. Lys-C protease (Roche Molecular Biochemicals, modified sequencing grade) was added to a final ratio of 1:10 (weight to weight), and the sample was incubated at 37 °C for 18 h.

Mass Spectrometry—MALDI-MS spectra were obtained with a Voyager DE-RI MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3-ns pulse). Spectra were collected in reflector mode. The accelerating voltage in the ion source was 20 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was α-cyano-4-hydroxyccinnamic acid dissolved in water/acetonitrile (1:1, v/v) to give a concentration of 5% formic acid, 50% acetonitrile, and 25% isopropanol. Spectra were collected in reflector mode. The accelerating voltage in the ion source was 20 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was α-cyano-4-hydroxyccinnamic acid dissolved in water/acetonitrile (1:1, v/v) to give a concentration of 5% formic acid, 50% acetonitrile, and 25% isopropanol. Each spectrum was produced by accumulating data using 128 laser pulses. Mass assignments were assigned with an accuracy of approximately ± 0.1% (± 1 Da/1000 Da). The computer program called Peptide Mass on the ExPaSy Molecular Biology Server was used to calculate the masses of all possible peptides.
FIG. 4. Detection of tryptic peptides derived from the cytoplasmic domain of β3 from AS-1 and AS-2 with MADLI-TOF mass spectrometry. A, the amino acid sequence of the cytoplasmic domain of β3 is shown along with the predicted cleavage points for trypsin (top...
Purification of the Active and Resting Forms of $\alpha_{IIb}\beta_3$ from Platelet Lysates—The ability to purify two forms of $\alpha_{IIb}\beta_3$ from platelet lysates is well documented in the literature (28, 29). Our procedure for obtaining the resting (AS-1) and active (AS-2) conformations of $\alpha_{IIb}\beta_3$ essentially paralleled the procedures established by others. With these methods, approximately 2.5 mg of AS-2 and 20 mg of AS-1 could be obtained from 50 units of outdated platelets. The purity of each form of the integrin was judged to be at least 90% by Coomassie staining of SDS-PAGE (Fig. 1A). We found no evidence of the reproducible co-purification of any low $M_r$ proteins that could be associated with $\alpha_{IIb}\beta_3$ in a stoichiometric manner.

One of the hallmark differences between the resting and active forms of $\alpha_{IIb}\beta_3$ on the platelet surface is the depth of the RGD binding pocket. This depth has been gauged by measuring the ability of resting and activated platelets to adhere to beads coupled to RGD peptides via spacers of varying length (39). To verify that the purified forms of $\alpha_{IIb}\beta_3$ are similar to the conformers observed on the platelet surface, we assessed the depth of the RGD binding pocket on AS-1 and AS-2. We measured the binding of each conformer to affinity columns in which the RGD motif was linked to the resin in different ways. In one approach, the RGD peptide was linked directly to the resin, providing a short spacer. In another approach, the RGD peptide was bridged to the resin with a hexanediamine spacer of about 18 Å. Only AS-2 bound the RGD peptide linked directly to Sepharose (Fig. 1B), but both AS-1 and AS-2 bound the beads in which the RGD was displayed on an extended linker. These observations indicate that the differences in the exposure of the RGD binding pocket on purified AS-1 and AS-2 are similar to those observed on the platelet surface.

Comparison of the Fibrinogen Binding Properties of AS-1 and AS-2—In equilibrium binding studies, Kouns et al. (28) found that only AS-2 could bind to Fg. No binding between AS-1 and Fg could be detected. We sought to determine whether the failure to detect binding between AS-1 and Fg is a consequence of a slow association rate or a rapid dissociation rate between the two proteins. This distinction has important implications for understanding how cellular signaling events regulate integrin activation.

SPR was used to measure $k_1$ for the binding of AS-1 and AS-2 to Fg (Fig. 2). The association between AS-1 and fibrinogen could not be detected, even at high concentrations of integrin. Under the conditions of this experiment, the lowest association rate that can be observed with the BIAcore was $1 \times 10^3$ m$^{-1}$ s$^{-1}$. Therefore, the rate at which AS-1 associates with Fg is below this value. In contrast, AS-2 bound to Fg with an association rate constant of $1.3 \times 10^5$ m$^{-1}$ s$^{-1}$. In related experiments, we found that the association rate between AS-2 and the model RGD-ligand Fab-9 was also about 7-fold faster than the rate of association between AS-1 and Fab-9 ($2.3 \times 10^5$ m$^{-1}$ s$^{-1}$ versus $3.2 \times 10^4$ m$^{-1}$ s$^{-1}$). Therefore, a primary difference between AS-1 and AS-2 is the rate at which ligands associate with their respective binding pockets.

Testing the Role of Mn$^{2+}$ as an Activator—The divalent ion Mn$^{2+}$ has been linked to the activation of integrins because it increases ligand binding affinity and promotes cell adhesion (20, 40, 41). It is widely thought that Mn$^{2+}$ mimics physiologic activation of integrin. To test this idea, we measured the ability of Mn$^{2+}$ to convert AS-1 to AS-2. AS-2 binds to Fg when Ca$^{2+}$ is the only available divalent ion, and this binding function is stable to prolonged dialysis. Therefore, these characteristics were set as criteria for the conversion of AS-1 to AS-2.

To perform the conversion test, AS-1, purified in Ca$^{2+}$-containing buffer, was dialyzed into buffer containing 200 μM Mn$^{2+}$. Then, the ability of the integrin to bind to fibrinogen was measured by SPR. As shown in Fig. 3, Mn$^{2+}$ increased the association between AS-1 and Fg to the point where it could be detected by SPR. However, when the Mn$^{2+}$-loaded AS-1 was returned to buffer containing only Ca$^{2+}$, its ability to bind Fg was lost. This cation-exchange procedure could be repeated through several cycles, each resulting in an increase in $k_1$ for Fg when Mn$^{2+}$ was present, and a subsequent ablation of binding to Fg when only Ca$^{2+}$ was present. Thus, whereas Mn$^{2+}$ increases the association rate of AS-1 for ligand, it does not convert AS-1 to AS-2.

Probing the Chemical Structure of AS-1 and AS-2 by Peptide Mass Fingerprinting—There is no detailed information on how the structures of AS-1 and AS-2 differ. This leaves open the possibility that they are alternatively spliced forms of the integrin or differ in some key posttranslational modification. A comprehensive mass fingerprinting study was undertaken to probe for differences between AS-1 and AS-2. Because of the accuracy of mass spectrometry, this type of analysis is capable of revealing differences in amino acid sequence and in post-translational modification. Mass fingerprinting was performed by separating the $\alpha_{IIb}$ and $\beta_3$ subunits (derived from AS-1 and arrows) and Lys-C protease (bottom arrows). The monoisotopic mass of the predicted fragments are shown above each peak. B, a portion of the mass spectra from $\beta_3$ derived from AS-1 and digested with trypsin is shown. The peptide peaks corresponding to each of the peptides from within the cytoplasmic tail are labeled (arrows). A smaller section of the spectra from $\beta_3$ derived from AS-1 but digested with Lys-C protease is shown in the inset, revealing a peptide fragment corresponding to the extreme C terminus of $\beta_3$. C, mass spectra derived from the AS-2 form of $\beta_3$ treated in the same manner as described for panel B are shown.
AS-2) by SDS-PAGE under reducing conditions. Bands corresponding to each subunit were excised and subjected to in-gel digestion with trypsin as described under "Materials and Methods." Then, samples were separated on 12% acrylamide gels, and peptide fragments were visualized by Coomassie staining. B, peptides 1, 7, and 9 generated by Asp-N digestion of αIIbβ3 were excised from the gel shown in panel A and subjected to in-gel tryptic digestion as described under "Materials and Methods." Their composition was determined by peptide mass fingerprinting with MALDI-TOF mass spectrometry. This figure contains structural information required to interpret results of these peptide mass fingerprints from peptides 1, 7, and 9 (A). The amino-terminal domain of the β3 subunit (boxed) is linked to the cysteine-rich domain (boxed) via a long-range disulfide bond between Cys-5 and Cys-435 (arrows). Other known disulfide bonds within the domain are also noted with arrows. Peptides 1, 7, and 9 were generated by cleavage with Asp-N protease, which cleaves on the amino-terminal side of aspartic acid residues. The Asp-N fragments were then excised from the gel and subjected to peptide mass fingerprinting using trypsin, which cleaves at lysine and arginine residues. Therefore, the cleavage points for both Asp-N and trypsin are shown as vertical lines above and below the amino acid sequence. In addition, the monoisotopic mass of all fragments that were detected with MALDI-TOF mass spectrometry is noted above the sequence. A key distinction between AS-1 and AS-2 is the presence of the tryptic peptide extending from residues 412–423 (bold text) in the mass fingerprints. This tryptic peptide is detected in mass fingerprints of Asp-N fragments 1 and 7 (AS-1) but is not detected in fragment 9 (AS-2). This tryptic peptide must be derived from the slightly larger Asp-N fragment, which was generated from the original digestion of αIIbβ3 and that extends Asp-393 to Asp-423 (underlined).
and AS-2 appeared to be identical, effort was focused on identifying the conformational distinctions between the two molecules. In one approach, we examined the sensitivity of disulfide bonds to reduction (Fig. 5). AS-1 was largely insensitive to mild reduction with DTT (Fig. 5A, lane 2). In contrast, the disulfide bond connecting the heavy and light chain of αIIbβ3 was reduced using only mild reductant (Fig. 5B, lane 2). Therefore, a major conformational distinction between AS-1 and AS-2 is the exposure of the disulfide bond joining the heavy and light chains of αIIb. With an increase in the amount of DTT, and with a prolonged incubation time, the disulfide bond connecting the heavy and light chain of αIIb in AS-1 was partially reduced (Fig. 5A, lane 3). The addition of RGD peptide stabilized both forms of the receptor to reducing agent (Fig. 5, A and B, lanes 4), indicating a conformational connection between the labile disulfide bonds and the ligand binding pocket.

Mapping Conformational Differences in the β3 Subunit of AS-1 and AS-2—To examine the conformational differences between AS-1 and AS-2 in more detail, the two proteins were subjected to a peptide mapping study. This procedure differed from the mass fingerprinting study because the proteins were subjected to limited digestion with Asp-N protease in solution. Then the resulting fragments were subjected to mass fingerprinting to determine their composition.

Peptide maps that revealed fragments that distinguish AS-1 and AS-2 were generated by limited proteolysis. The Asp-N fragments unique to the digests of AS-1 and AS-2 were evident on SDS-PAGE (Fig. 6A). Each of the unique Asp-N fragments was excised from the SDS gel and subjected to in-gel digestion with trypsin and peptide mass fingerprinting as described under “Materials and Methods.” From the masses of the peptides present in these MALDI spectra, the composition of these peptides and their location within the sequences of αIIb and β3 was surmised. The AS-N-generated fragments can be separated into two groups that are discussed below.

Table II: Mass fingerprints of fragments 1, 7, and 9 derived from β3

| Peak no. | Predicted mass | Observed mass | Corresponding position in β3 (residues) |
|----------|----------------|---------------|----------------------------------------|
| 1        | 918.5          | 918.6         | 1–8                                    |
| 2        | 1054.6         | 1055.2        | 27–37                                  |
| 3        | 1123.7         | 1123.8        | 413–422                                |
| 4        | 1296.6         | 1297.3        | 423–433                                |
| 5        | 1643.7         | 1642.6        | 434–448                                |
| 6        | 1820.8         | 1821.3        | 47–62                                  |
| 7        | 2187.9         | 2186.4        | 9–26                                   |

AS-1 and AS-2 were digested with Asp-N protease, and the resulting fragments were separated on SDS-PAGE. Peptide fragments 1 and 7 that are unique to AS-1 (Figure 6A) and fragment 9 that is unique in AS-2 were excised and subjected to in-gel trypsin digestion to determine their composition. The mass fingerprint of each fragment was determined using MALDI-TOF mass spectrometry. The mass range extending from 500 to 6000 Da was examined. The predicted mass of each peptide and the corresponding fragment observed in MALDI are shown. Some tryptic peptides fall outside of the detectable mass range. Consequently, the peptides that are observed do not make up the contiguous sequence of each Asp-N fragment.

A Difference in the Sensitivity of Disulfide Bonds in AS-1 and AS-2 to Reduction—Given that the chemical structure of AS-1 and AS-2 are identical, effort was focused on identifying the conformational distinctions between the two molecules. In one approach, we examined the sensitivity of disulfide bonds to reduction (Fig. 5). AS-1 was largely insensitive to mild reduction with DTT (Fig. 5A, lane 2). In contrast, the disulfide bond connecting the heavy and light chain of αIIb was reduced using only mild reductant (Fig. 5B, lane 2). Therefore, a major conformational distinction between AS-1 and AS-2 is the exposure of the disulfide bond joining the heavy and light chains of αIIb. With an increase in the amount of DTT, and with a prolonged incubation time, the disulfide bond connecting the heavy and light chain of αIIb in AS-1 was partially reduced (Fig. 5A, lane 3). The addition of RGD peptide stabilized both forms of the receptor to reducing agent (Fig. 5, A and B, lanes 4), indicating a conformational connection between the labile disulfide bonds and the ligand binding pocket.

Mapping Conformational Differences in the β3 Subunit of AS-1 and AS-2—To examine the conformational differences between AS-1 and AS-2 in more detail, the two proteins were subjected to a peptide mapping study. This procedure differed from the mass fingerprinting study because the proteins were subjected to limited digestion with Asp-N protease in solution. Then the resulting fragments were subjected to mass fingerprinting to determine their composition.

Peptide maps that revealed fragments that distinguish AS-1 and AS-2 were generated by limited proteolysis. The Asp-N fragments unique to the digests of AS-1 and AS-2 were evident on SDS-PAGE (Fig. 6A). Each of the unique Asp-N fragments was excised from the SDS gel and subjected to in-gel digestion with trypsin and peptide mass fingerprinting as described under “Materials and Methods.” From the masses of the peptides present in these MALDI spectra, the composition of these peptides and their location within the sequences of αIIb and β3 was surmised. The AS-N-generated fragments can be separated into two groups that are discussed below.

Table II: Mass fingerprints of fragments 1, 7, and 9 derived from β3

| Peak no. | Predicted mass | Observed mass | Corresponding position in β3 (residues) |
|----------|----------------|---------------|----------------------------------------|
| 1        | 918.5          | 918.6         | 1–8                                    |
| 2        | 1060.5         | 1060.9        | 490–498                                |
| 3        | 1123.7         | 1123.9        | 413–422                                |
| 4        | 1296.6         | 1296.8        | 423–433                                |
| 5        | 1501.5         | 1510.4        | 520–530                                |
| 6        | 1643.7         | 1642.7        | 434–448                                |
| 7        | 1658.7         | 1658.7        | 564–578                                |
| 8        | 1798.7         | 1797.7        | 550–563                                |
| 9        | 1820.8         | 1821.3        | 47–62                                  |
| 10       | 2000.7         | 2000.7        | 499–515                                |
| 11       | 2921.3         | 2921.3        | 423–447                                |
| 12       | 3223.3         | 3222.3        | 9–37                                   |

To examine the conformational differences between AS-1 and AS-2 in more detail, the two proteins were subjected to a peptide mapping study. This procedure differed from the mass fingerprinting study because the proteins were subjected to limited digestion with Asp-N protease in solution. Then the resulting fragments were subjected to mass fingerprinting to determine their composition.

Peptide maps that revealed fragments that distinguish AS-1 and AS-2 were generated by limited proteolysis. The Asp-N fragments unique to the digests of AS-1 and AS-2 were evident on SDS-PAGE (Fig. 6A). Each of the unique Asp-N fragments was excised from the SDS gel and subjected to in-gel digestion with trypsin and peptide mass fingerprinting as described under “Materials and Methods.” From the masses of the peptides present in these MALDI spectra, the composition of these peptides and their location within the sequences of αIIb and β3 was surmised. The AS-N-generated fragments can be separated into two groups that are discussed below.

Table II: Mass fingerprints of fragments 1, 7, and 9 derived from β3

| Peak no. | Predicted mass | Observed mass | Corresponding position in β3 (residues) |
|----------|----------------|---------------|----------------------------------------|
| 1        | 918.5          | 918.6         | 1–8                                    |
| 2        | 1060.5         | 1060.5        | 490–498                                |
| 3        | 1123.7         | 1123.9        | 413–422                                |
| 4        | 1296.6         | 1296.8        | 423–433                                |
| 5        | 1501.5         | 1510.4        | 520–530                                |
| 6        | 1643.7         | 1642.7        | 434–448                                |
| 7        | 1658.7         | 1658.7        | 564–578                                |
| 8        | 1798.7         | 1797.7        | 550–563                                |
| 9        | 1820.8         | 1821.3        | 47–62                                  |
| 10       | 2000.7         | 2000.7        | 499–515                                |
| 11       | 2921.3         | 2921.3        | 423–447                                |
| 12       | 3223.3         | 3222.3        | 9–37                                   |

spectra containing tryptic peptides derived from the tail of β3 are shown in Fig. 4. In fingerprints generated with trypsin, peptides encompassing β3 residues 717 to 762, all but the two most C-terminal amino acids, were observed in MALDI profiles (Fig. 4B). The presence of the carboxyl-terminal two residues on β3 was confirmed by digestion with Lys-C protease, yielding the predicted fragment with mass of 1561 daltons (Fig. 4, B and C, insets). Phosphorylation of the cytoplasmic tails would be evident as an increase in the mass of tryptic peptides by 80 daltons per phosphate group. There was no reproducible evidence of phosphorylation of the peptides derived from either AS-1 or AS-2. This observation was supported by the inability to Western blot either form of AS-1 or AS-2. This observation was supported by the inability to detect the peptides derived from either AS-1 or AS-2.
TABLE III

Mass fingerprints of fragments 2–6 and 8 derived from αIIb

| Peak no. | Predicted mass | Observed mass | Corresponding position in αIIb (residues) |
|----------|----------------|---------------|-----------------------------------------|
| 1        | 1035.6         | 1035.7 ± 0.1  | 328–335                                 |
| 2        | 1331.7         | 1331.8 ± 0.2  | 356–368                                 |
| 3        | 1502.8         | 1502.6 ± 0.1  | 387–400                                 |
| 4        | 1588.8         | 1587.4 ± 0.3  | 304–317                                 |
| 5        | 1779.8         | 1779.9 ± 0.1  | 369–386                                 |
| 6        | 2022.1         | 2022.3 ± 0.2  | 336–355                                 |
| 7        | 2311.0         | 2310.2 ± 0.1  | 282–303                                 |
| 8        | 2353.2         | 2354.1 ± 0.1  | 401–422                                 |
| 9        | 2925.4         | 2926.1 ± 0.2  | 423–449                                 |

Section B: fragment 5; derived from αIIb

| Peak no. | Predicted mass | Observed mass | Corresponding position in αIIb (residues) |
|----------|----------------|---------------|-----------------------------------------|
| 1        | 1035.6         | 1035.7        | 328–335                                 |
| 2        | 1331.7         | 1331.8        | 356–368                                 |
| 3        | 1502.8         | 1502.6        | 387–400                                 |
| 4        | 1588.8         | 1587.4        | 304–317                                 |
| 5        | 1779.8         | 1779.9        | 369–386                                 |
| 6        | 1999.1         | 1999.6        | 456–471                                 |
| 7        | 2311.0         | 2310.2        | 282–303                                 |
| 8        | 2353.2         | 2354.1        | 401–422                                 |
| 9        | 2925.4         | 2926.1        | 423–449                                 |

Section C: fragment 6; derived from αIIb

| Peak no. | Predicted mass | Observed mass | Corresponding position in αIIb (residues) |
|----------|----------------|---------------|-----------------------------------------|
| 1        | 1035.6         | 1035.7        | 328–335                                 |
| 2        | 1331.7         | 1331.8        | 356–368                                 |
| 3        | 1502.8         | 1502.6        | 387–400                                 |
| 4        | 1588.8         | 1587.4        | 304–317                                 |
| 5        | 1779.8         | 1779.9        | 369–386                                 |
| 6        | 1999.1         | 1999.6        | 456–471                                 |
| 7        | 2311.0         | 2310.2        | 282–303                                 |
| 8        | 2353.2         | 2354.1        | 401–422                                 |
| 9        | 2925.4         | 2926.1        | 423–449                                 |

Section D: fragment 7; derived from αIIb

| Peak no. | Predicted mass | Observed mass | Corresponding position in αIIb (residues) |
|----------|----------------|---------------|-----------------------------------------|
| 1        | 1035.6         | 1035.7        | 328–335                                 |
| 2        | 1331.7         | 1331.8        | 356–368                                 |
| 3        | 1502.8         | 1502.6        | 387–400                                 |
| 4        | 1588.8         | 1587.4        | 304–317                                 |
| 5        | 1779.8         | 1779.9        | 369–386                                 |
| 6        | 1999.1         | 1999.6        | 456–471                                 |
| 7        | 2311.0         | 2310.2        | 282–303                                 |
| 8        | 2353.2         | 2354.1        | 401–422                                 |
| 9        | 2925.4         | 2926.1        | 423–449                                 |

CONCLUSION

Despite advances in our understanding of the signaling pathways that enact activation of integrins, little attention has been given to the changes in the kinetics of ligand binding that lead to activation. Similarly, the identity of chemical modifications, or positions of conformational change, that accompany activa-
tion have not been mapped. Here, we address these issues by using the platelet integrin αIIbβ3 as a model.

As an initial step toward understanding the differences between AS-1 and AS-2, we compared their ligand binding properties. Prior study shows that αIIbβ3 contains two ligand binding sites, one for Fg and another site that binds to RGD (35, 45). These ligand binding sites are physically separate but are linked kinetically. For example, RGD ligand blocks the association of Fg with its binding site and has the ability to dissociate pre-bound Fg (35). Our finding that the RGD binding site is present on AS-1 and AS-2 confirms the prior observation by Kouns (28). The fact that AS-1 and AS-2 have RGD binding sites with different degrees of exposure, or of depth, is also consistent with the observation that the depth of this site is regulated by activation on the platelet surface (39). The fact that the RGD site is more accessible to ligand on AS-2 also provides a structural basis for the more rapid rate of association with the model RGD ligand Fab-9.

No study has measured the association and dissociation rates between AS-1 and Fg. Therefore, the lack of binding between AS-1 and Fg under equilibrium conditions could have resulted from a high dissociation rate rather than a lack of association. In the present study, no association between AS-1 and Fg could be detected, even at very high concentrations of Fg. This observation suggests that the Fg binding site may be entirely absent on resting integrin and may come to exist only when conformational rearrangements create such a site. This is in contrast to the RGD binding site, which can be detected on both AS-1 and AS-2 and which appears to be displayed at different depths on the two conformers.

Many artificial stimuli are used to mimic cellular activation in the study of integrin activation. Among these are antibodies that alter the integrins affinity state, reducing agents like dithiothreitol and the divalent ion Mn2+. Mn2+ has been used extensively as a stimulus because it binds to integrins and increases the ligand association rate (20, 40, 41). Because of this property, Mn2+ has come to be considered a “universal” integrin activator. Given this prevailing notion, we suspected that Mn2+ might be able to convert AS-1 to AS-2. The conformations of AS-1 and AS-2 are stable to prolonged dialysis, so we were able to perform a series of experiments to test the ability of Mn2+ to convert AS-1 to AS-2. The two parameters that were considered as benchmarks of conversion were 1) the ability to bind to Fg in the presence of Ca2+, and 2) the stability of this property to dialysis. Dialysis of AS-1 into buffer containing Mn2+ increased the rate of association for Fg, but this effect was lost when the integrin was dialyzed back into buffer containing Ca2+. Therefore, we conclude that Mn2+ enhances the rate of association with ligand but fails to induce the conformational changes that are equivalent to activation. We have also observed that treatment of AS-1 with mild reductant, like DTT, will increase the rate of association with ligand. Yet, our observations indicate that DTT also fails to convert AS-1 to AS-2.2

Prior study established purification protocols for AS-1 and AS-2, but no detailed comparison of their chemical structures had been performed. This left open the possibility that the two variants of αIIbβ3 were actually alternatively spliced forms of the integrin, especially because alternative splicing has been observed for both αIIb and for β3 (46–48). By using peptide mass fingerprinting, we were able to detect tryptic peptide fragments that constitute more than 85% of the sequence of αIIb and β3 in both the AS-1 and AS-2 forms. Most of the peptides that were not detected by MALDI were of very low mass. These studies showed the amino acid sequence of the two proteins to be virtually identical, and indicate that AS-2 is not an alternatively spliced form of the integrin present in platelets at low abundance.

We anticipated that the mass fingerprinting study would reveal some type of post-translational modification that would account for the difference in activity of AS-1 and AS-2. Of particular interest were potential phosphorylations within the cytoplasmic domain of β3. This subunit contains two consensus tyrosine phosphorylation sites and several serine and threonine residues that are candidates for modification by phosphate. We interpret the lack of phosphorylation on AS-1 or AS-2 to show that phosphorylation is not necessary to maintain the integrin in an active and stable conformation. We cannot exclude the possibility that phosphorylation within the cytoplasmic domains provides the activation energy to guide the integrin through a transition state that ultimately “decays” to a stable conformation like AS-2. There is certainly precedent for stepwise activation because some platelet agonists induce reversible activation of αIIbβ3 but others cause an irreversible activation (49, 50). Similarly, the activation of the αIIbβ3 integrin is a two-stage event (51).

Even though the chemical structure of AS-1 and AS-2 are virtually identical, they have significant differences in conformation. One conformational difference centered around the disulfide bond that connects the heavy and light chain of αIIb. Within AS-2, this disulfide bond is sensitive to mild reducing conditions, whereas the same bond in AS-1 is stable. Interestingly, the binding of RGD ligand stabilizes this bond to reduction, providing a conformational link to the RGD binding site. The association of this segment of the integrin with modulation of ligand binding affinity is consistent with prior work showing that monoclonal antibodies that bind in this vicinity can activate the ligand binding function of αIIbβ3 (52). It has also been reported that the cleavage of αIIb in this region by neutrophil elastase can induce platelet aggregation (19). The association of the junction between the heavy and light chain of αIIb with activation is also of interest because the junction is generated by intracellular proteolysis during maturation of the integrin. Most integrin α subunits undergo similar processing at dibasic residues within the α subunits. Although this cleavage is not required for ligand binding, it may be important for activation. Mutations in the integrin α gene that eliminate the proteolytic processing site render the αβ integrin resistant to activation and kept the integrin in a low affinity state on the cell surface (53). Along with the differences that AS-1 and AS-2 display in this region of the integrin, all of these observations strongly implicate the domain encompassing the junction of the heavy and light chains of integrin α subunits as a key site where conformational changes accompany activation.

Peptide mapping also showed that AS-1 and AS-2 exhibit a difference in conformation in a region of the β3 subunit that can be considered a disulfide “knot” connecting three regions of the protein that are well separated in the linear sequence. These are the extreme amino terminus (residues 1 to 62), the cysteine-rich domain (roughly residues 420 to 620), and a region proximal to the membrane spanning segment (residues 635 to 663). Within this knotted domain, a primary difference between AS-1 and AS-2 appears to center on a peptide extending from residues 393 to 423 that appears to be exposed in AS-2 and buried in AS-1. This segment of β3 is directly adjacent to Cys-435 which forms the disulfide bond to Cys-5, and it encompasses Cys-405 which links to Cys-655. Interestingly, the epitopes for many LIBS antibodies, which can activate the integrin and induce platelet aggregation, bind to these domains (54, 55). Consequently, the evidence linking conformational

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2 B. Yan and J. Smith, forthcoming report.
changes within the disulfide knotted core of $\beta_2$ to the process of activation is compelling.

Within the limits of detection, the resting and active forms of $\alpha_{IIb}\beta_2$ appear to have the same chemical structure. However, the two conformers have significant differences in conformation. These conformational differences primarily effect the rate of ligand association and, in fact, may create of the Fg binding site. These findings suggest that future effort should focus on understanding the mechanism by which structural transitions within the ectodomain of the integrin are brought about by inside-out signaling.

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