Redox Modulation of Integrin $\alpha_{IIb}\beta_3$ Involves a Novel Allosteric Regulation of Its Thiol Isomerase Activity†

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ABSTRACT: The molecular mechanisms involved in regulating the activation-dependent conformational switch in integrins are not known although recent evidence suggests that integrins are a direct target for redox modulation. We have identified an endogenous integrin thiol isomerase activity that may be responsible for regulating integrin activation states. The purpose of this study was to examine the effects of redox conditions elicited by nitric oxide and glutathione on the thiol isomerase activity of the platelet integrin $\alpha_{IIb}\beta_3$ and also on the activation status of this integrin in intact platelets. The universal integrin activator, Mn^{2+}, stimulates the thiol isomerase activity in purified $\alpha_{IIb}\beta_3$. Kinetic analysis reveals that $\alpha_{IIb}\beta_3$ is an allosteric enzyme which displays positive cooperativity in the presence of Mn^{2+} with an apparent Hill coefficient of 1.9. Also, addition of Mn^{2+} to platelets results solely in activation of the integrin as demonstrated by the binding of the antibody PAC-1. The addition of the nitric oxide donors SNP, SIN-1, and SNOAC in combination with glutathione can directly reverse the activation state of the platelet integrin induced by Mn^{2+}. These compounds have no effect on platelet secretory responses indicating a direct effect on the integrin. In the presence of nitric oxide and glutathione, the enzymatic activity of $\alpha_{IIb}\beta_3$ also displays positive cooperativity (apparent Hill coefficient of 1.9), and a significant increase in the saturability of the enzyme was observed. Thus, redox agents simultaneously modulate the thiol isomerase activity of purified $\alpha_{IIb}\beta_3$ and its active conformation in intact platelets, suggesting a molecular mechanism for integrin regulation.

Integrins are heterodimeric transmembrane cell-adhesion molecules consisting of α and β subunits. The recognition and binding of integrins to their ligands are controlled by alterations in their affinity state associated with conformational changes. The platelet-specific integrin $\alpha_{IIb}\beta_3$ is the most studied of all integrins as it is a key player in the pathogenesis of thrombotic disorders. Therefore, understanding its regulation is of the utmost importance in developing new therapies to combat diseases such as unstable angina, myocardial infarction, or stroke. On resting, unactivated platelets this receptor has a very low affinity for its primary ligand fibrinogen. Following platelet activation, $\alpha_{IIb}\beta_3$ is converted to a high-affinity receptor for fibrinogen although the precise nature of the molecular switch that converts it to this state is not known.

Integrins are cysteine-rich proteins. Changes in integrin conformation appear to be dependent on altered disulfide-bonding patterns between the many cysteine residues, as thiol-modifying reagents such as DTT[1] can induce high-affinity ligand binding (*1*−*3*). Alanine replacement of certain cysteine residues such as cysteine-5 or cysteine-435 in the β_3 subunit results in a constitutively activated integrin, suggesting that the long-range disulfide bond which involves these two residues is critical in determining high-affinity ligand binding (*4*, *5*). Moreover, in contrast to the original reports by Calvete et al. (*6*, *7*), it is now known that free thiols exist in $\alpha_{IIb}\beta_3$ and that these alter in response to integrin activation (*8*). Many antibody markers of integrin activation map to the cysteine-rich EGF-like domains of the β subunit (*9*), and point mutations in this region result in an integrin locked in a high-affinity state (*5*).

We have demonstrated that integrins, including $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$, have an endogenous thiol isomerase activity. This activity was predicted from the presence of a highly conserved Cys-Gly-X-Cys (CGXC) motif in each of the β-subunit EGF-like domains (*10*). This motif is common to the active sites of other thiol-modifying enzymes such as thioredoxin and protein disulfide isomerase (*11*) and may be responsible for autoregulation of the integrin affinity state. Recently, the importance of disulfide metabolism in platelet function has been highlighted by the demonstration that

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[1] Abbreviations: cCMP, cytidine 2′,3′-cyclic monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; Mn^{2+}, manganese cation; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NO, nitric oxide; PE, phycoerythrin; PGE_1, prostaglandin E_1; rdRNase, reduced and denatured RNase A; SIN-1, 3-morpholinosydnonimine; SNOAC, *S*-nitroso-*N*-acetylcysteine; SNP, sodium nitroprusside.

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glutathione (GSH) redox potentials can regulate the activation state of the platelet and also alter sulfhydryl exposure in $\alpha_{IIb}\beta_3$ (*12*). In addition, the activation state of purified $\alpha_{IIb}\beta_3$ in a cell-free system can be altered in a redox-dependent manner by a combination of nitric oxide (NO) and GSH (*8*). In this study we attempt to analyze parallel changes in integrin activation status in intact platelets and enzymatic thiol isomerase activity in purified preparations of $\alpha_{IIb}\beta_3$ under equivalent experimental conditions in order to establish an interdependence of these two parameters.

NO is an important biological molecule with a multitude of functions in the cardiovascular system (*13*). It participates in many responses including platelet regulation, vasodilation, cell toxicity, and neurotransmission (*14*). Recent studies indicate that NO directly modifies protein function independent of cGMP production by specifically targeting cysteinyl thiols and transition metal centers. In the majority of cases the protein function is regulated by a nitrosylation modification of a single critical cysteine residue within an acid−base or structural motif. Furthermore, these cysteines may also be subject to GSH-dependent modification (*15*).

Here we show that the enzymatic thiol isomerase activity of purified integrin $\alpha_{IIb}\beta_3$ is induced in response to manganese cations (Mn^{2+}), a known integrin regulator, through an unknown mechanism. This enzymatic activity is altered by NO and GSH. In parallel, these redox agents reverse the Mn^{2+}-induced activated conformation of $\alpha_{IIb}\beta_3$ in the intact platelet. These findings provide a direct link between redox biology and platelet function and are supportive of a functional role for the endogenous thiol-modifying activity in the integrin.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal antibodies fluorescein isothiocyanate-labeled PAC-1 (PAC-1-FITC) and phycoerythrin-labeled CD62P (CD62P-PE) were obtained from Becton Dickinson, Oxford, England. Ribonuclease (RNase) A, human $\alpha_{IIb}\beta_3$, guanidine hydrochloride, and 3-morpholinosydnonimine (SIN-1) were purchased from CN Biosciences U.K. Ltd., Nottingham, U.K. Sodium nitroprusside (SNP) was obtained from BDH Chemicals Ltd., Poole, England. Micro-bio-spin 6 columns were obtained from Bio-Rad Laboratories Ltd., Hemel Hempstead, England. Bovine serum albumin, cytidine 2′,3′-cyclic monophosphate (cCMP), dithiothreitol (DTT), 3-(*N*-morpholino)propanesulfonic acid (MOPS), reduced glutathione (GSH), PD-10 Sephadex G-25 columns, Sepharose 2B-300, sodium nitrite, thrombin, Triton X-100, and other general reagents were obtained from Sigma Aldrich Ltd., Tallaght, Ireland.

Thiol Isomerase Activity Assay. Reduced and denatured RNase A (rdRNase A) was prepared as previously described (*10*). Briefly, RNase A (30 mg) was incubated overnight with 0.15 M DTT and 6 M guanidine hydrochloride in 1.5 mL of 0.1 M Tris-HCl, pH 8.6, at room temperature. rdRNase was desalted on a Sephadex G-25 column equilibrated with 0.01 N HCl. Fractions were analyzed spectrophotometrically at 275 nm, and concentrations were determined using an extinction coefficient of 9200 M^{-1} cm^{-1}. Fractions of the highest concentrations were aliquoted and stored under argon at −80 °C until use in experiments. Purified $\alpha_{IIb}\beta_3$ (1.5 μM) was diluted into 0.1 M Tris, pH 7.4, with either 1 mM EDTA or 1 mM $MnCl_2$. The rdRNase was added at a final concentration of 30 μM. The extent of rdRNase reactivation was measured after a 48 h incubation by monitoring changes in absorbance at 284 nm using 0.44 mM cCMP in 0.1 M MOPS, pH 7.0, as substrate for RNase using a Helios split beam spectrophotometer.

Determination of Enzymatic Parameters. Purified $\alpha_{IIb}\beta_3$ (1.5 μM) was incubated with and without a combination of 10 μM SNP and 30 μM GSH in the presence of 0.1 M Tris, pH 7.4, with 1 mM $MnCl_2$ for 5 min at room temperature. Samples were centrifuged at 1000*g* for 4 min through micro-bio-spin 6 columns equilibrated with the Tris/$MnCl_2$ buffer to separate the redox-modified $\alpha_{IIb}\beta_3$ from excess GSH and SNP. RdRNase ranging in concentrations from 0 to 120 μM was added to the modified $\alpha_{IIb}\beta_3$. The amount of active RNase was determined by measuring the change in absorbance at 284 nm after a 48 h incubation, as described previously. Kinetic parameters were determined by nonlinear regression using Sigmaplot 8. Data gave sigmoidal plots of substrate concentration versus initial velocity, and linear Hill plots were generated.

Preparation of Gel-Filtered Platelets. Venous whole blood was drawn from normal healthy volunteers free from any drugs that may affect platelet function for 7−10 days. Blood was drawn from the antecubital vein using a Butterfly 19 gauge needle and anticoagulated into 0.15 volume of acid−citrate−dextrose (ACD: 38 mM citric acid, anhydrous, 75 mM sodium citrate, 124 mM dextrose) (*16*). Blood was centrifuged at 170*g* for 12 min at room temperature. Platelet-rich plasma (PRP) was removed to a separate plastic container, acidified to pH 6.5 with ACD, and PGE_1 (1.5 μM) was added. The platelets were pelleted through the plasma by centrifugation at 1000*g* for 12 min at room temperature. The supernatant was removed, and the platelets were resuspended in buffer A (130 mM NaCl, 10 mM trisodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris, pH 7.35). The resuspended platelets were applied to a packed Sepharose 2B-300 column (5−6 mL of packed Sepharose/mL of applied platelets in a column 1.5 cm in diameter) equilibrated with buffer A and filtered. Fractions (0.5 mL) were collected, and the platelet count in each fraction was assessed using a Sysmex K-1000 counter (Toa Medical Electronics Co. Ltd., Kobe, Japan). The fractions with the highest platelet count were pooled, and calcium chloride ($CaCl_2$) at a final concentration of 1.8 mM was added.

Flow Cytometry. Dual-labeled flow cytometry was used to determine the activation state of both the platelet and the integrin $\alpha_{IIb}\beta_3$. Platelet activation was monitored using a phycoerythrin- (PE-) conjugated monoclonal antibody directed against P-selectin (CD62P), which is released from the α-granule and expressed on the platelet surface upon activation. Integrin activation was monitored using PAC-1-FITC, a monoclonal antibody directed against an active conformation of $\alpha_{IIb}\beta_3$. All experiments were carried out at room temperature on a Becton-Dickinson FACScan flow cytometer at 488/510 nm. Data acquisition and analysis were performed with the Cell Quest program. Platelet populations were gated, and mean fluorescece histograms were generated for each sample. Statistical analysis was performed on the geometric scale.

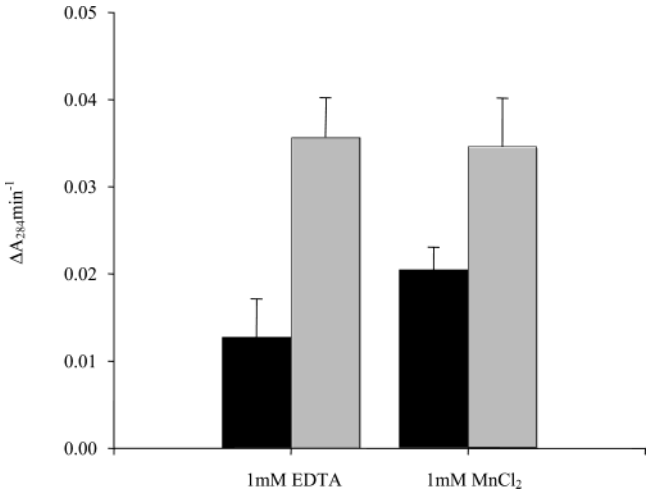

FIGURE 1: Mn^{2+} stimulates the thiol isomerase activity of $\alpha_{IIb}\beta_3$. The thiol isomerase activity of $\alpha_{IIb}\beta_3$ was measured in the presence of either 1 mM EDTA or 1 mM $MnCl_2$, a known integrin activator. This activity is quantified by measuring the hydrolysis of cCMP, the substrate for refolded rdRNase at 284 nm. Refolding of rdRNase occurs in the presence of 1.5 μM $\alpha_{IIb}\beta_3$, a source of thiol isomerase activity, after a 48 h incubation. The graph shows the difference between the observed RNase activity in the absence (solid bar) and presence (shaded bar) of purified $\alpha_{IIb}\beta_3$. Mn^{2+} stimulates the thiol isomerase activity of $\alpha_{IIb}\beta_3$ to levels comparable to those obtained in the presence of EDTA, with no statistical differences observed between the two treatments. Results shown are the mean of four experiments ± SEM.

Sample Preparation for Flow Cytometry. Gel-filtered platelets were diluted 1:1 in buffer A and activated with either 1 unit/mL thrombin, a potent physiological platelet agonist, or 1 mM $MnCl_2$, a selective integrin activator, for 3 min at 37 °C. Once activated, platelets were incubated with 3 mM GSH alone, with one of the following NO donors, 1 mM SNP, 10 μM SIN-1, or 10 μM SNOAC, or with a combination of GSH and NO for 5 min at room temperature. An array of NO donors, each of which are known to have slightly different properties, was used to ensure the specificity of the effect. PAC-1 and CD62P antibodies were added to the platelets for 10 min. Samples were fixed in 1% formaldehyde and analyzed in a flow cytometer at 488/510 nm at room temperature. Platelet populations were gated, counting 10000 events, and mean fluorescence histograms were generated for each sample.

Statistics. The statistical analysis performed was either the 2 factor or 3 factor analysis of variance (ANOVA). The 2 factor ANOVA was followed by a series of Student *t*-tests.

RESULTS

Effect of Mn^{2+} on the Thiol Isomerase Activity of $\alpha_{IIb}\beta_3$. The ability of the thiol isomerase activity of $\alpha_{IIb}\beta_3$ to catalyze the reactivation and renaturation of rdRNase A was determined by incubating rdRNase in an EDTA buffer containing $\alpha_{IIb}\beta_3$. After a period of 48 h the recovered RNase A was added to its substrate cCMP. The increase in the activity of RNase A was observed as a continual increase in the rate of cCMP hydrolysis at 284 nm over time. As we have demonstrated previously, EDTA maximally stimulates the thiol isomerase activity of the purified integrin. In the presence of Mn^{2+} there was a similar stimulatory effect on the thiol isomerase activity of $\alpha_{IIb}\beta_3$. The level of activity observed in the presence of Mn^{2+} was comparable to that

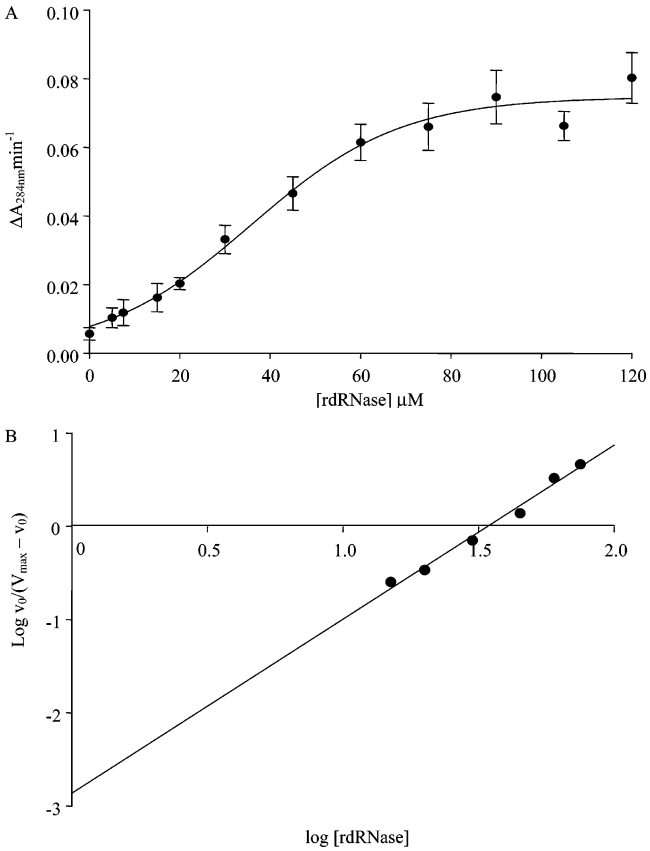

FIGURE 2: $\alpha_{IIb}\beta_3$ displays positive cooperativity in the presence of Mn^{2+}. (A) The thiol isomerase activity of $\alpha_{IIb}\beta_3$ was measured in the presence of 1 mM $MnCl_2$ using increasing concentrations of rdRNase (0−120 μM). The assay was performed after a 48 h incubation with 1.5 μM $\alpha_{IIb}\beta_3$. Nonlinear regression reveals a sigmoidal regression curve with $r^2 = 0.99$. Data shown are the mean of five experiments ± SEM. (B) Data were transformed into a Hill plot of log [rdRNase] vs log $v_0/(V_{max} - v_0)$. The Hill coefficient $n_H = 1.9$, indicating positive cooperativity, and $K_{0.5} = 34$ μM, with $r^2 = 0.99$.

obtained when the assay was performed in the EDTA buffer with no significant statistical difference between the two treatments (Figure 1).

The kinetic parameters of the thiol isomerase activity of $\alpha_{IIb}\beta_3$ were determined in the presence of Mn^{2+} by measuring its enzymatic activity as a function of increasing rdRNase concentration. Nonlinear regression revealed a sigmoidal fit with $r^2 = 0.99$. This demonstrates for the first time the allosteric nature of the enzymatic activity of the integrin, indicating the presence of regulatory sites which bind substrate in a cooperative manner (Figure 2A). Linear transformation of the 10−90% data into a Hill plot of log [rdRNase] vs log $v_0/(V_{max} - v_0)$ yielded a straight line with $r^2 = 0.99$. The slope of the line, which corresponds to the apparent Hill coefficient n_H, was determined to be 1.9. Since this value is greater than 1, it indicates positive cooperativity. The $K_{0.5}$ has a value of 34 μM, which represents the substrate concentration which gives a velocity equal to half the maximal velocity of the enzyme (Figure 2B).

Differential Effects of Thrombin and Mn^{2+} in Whole Platelets. Activation of platelets with thrombin (1 unit/mL) causes an increase in both PAC-1 and CD62P binding, markers of integrin and platelet activation, respectively. In contrast, addition of $MnCl_2$ (1 mM) to platelets results in increased binding of PAC-1 but has no stimulatory effect

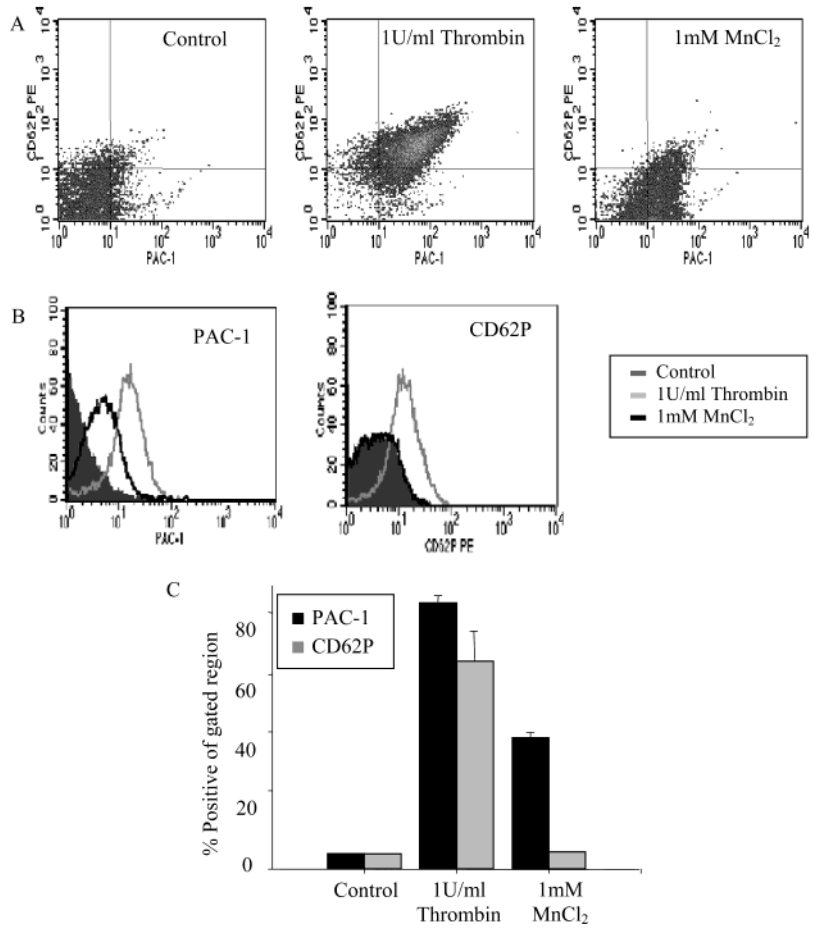

FIGURE 3: Thrombin and manganese differentially activate platelets. (A) Representative flow cytometry density plots showing control, thrombin-activated (1 unit/mL), and Mn^{2+}-activated (1 mM) gel-filtered platelets labeled with PAC-1, a marker of activated $\alpha_{IIb}\beta_3$, and CD62P, a marker of platelet secretion. (B) shows thrombin (gray line) causes an increase in both PAC-1 and CD62P expression, while Mn^{2+} (black line) increases PAC-1 expression with no increase in CD62P mean fluorescence. (C) shows the mean of three separate experiments demonstrating that Mn^{2+} can specifically modulate integrin activation, independent of platelet secretion.

on CD62P expression. The level of PAC-1 binding to Mn^{2+}-treated platelets is approximately 50% of that observed in platelets activated with thrombin. This reflects the fact that thrombin, a very potent platelet agonist, maximally activates both the integrin and the platelet with subsequent release of $\alpha_{IIb}\beta_3$ from internal platelet stores. In contrast, Mn^{2+} has been shown to increase the ligand association rate of $\alpha_{IIb}\beta_3$ (*17*), and therefore, Mn^{2+} specifically induces an active-like conformational change in $\alpha_{IIb}\beta_3$. This change allows the PAC-1 antibody to bind to the integrin but has no effect on platelet activation. Therefore, in the presence of Mn^{2+} the platelet is maintained in a quiescent state, as there is no detection of CD62P on the surface of the platelet. Mn^{2+} can therefore specifically modulate the integrin activation state in intact platelets, independent of platelet activation (Figure 3). Thus Mn^{2+} can stimulate the thiol isomerase activity of purified $\alpha_{IIb}\beta_3$ and can selectively induce conformational change(s) in the integrin in situ in the platelet.

NO and GSH Reverse the Activation State of $\alpha_{IIb}\beta_3$ in Intact Platelets. The NO donors SIN-1 and SNOAC, in the absence of GSH, reversed PAC-1 binding to platelets already treated with Mn^{2+}, indicating a direct NO effect on the integrin. GSH alone also significantly reversed PAC-1 binding to the Mn^{2+}-treated platelets, though to a greater extent than the NO donors alone. This effect was accentuated significantly in the presence of the NO donor SNP and was therefore deemed to be additive. There was no observed surface expression of CD62P in the Mn^{2+}-treated platelets, which remained at levels observed in control platelets at all times (Figure 4). A similar reversal in PAC-1 binding with no effect on CD62P expression was observed upon addition of GSH and NO donors to platelets maximally activated with thrombin (1 unit/mL) (data not shown). Thus, treatment of preactivated platelets with a combination of NO and GSH resulted in a highly significant reduction of PAC-1 binding to the integrin, indicating a reversal of the activation state of $\alpha_{IIb}\beta_3$.

NO and GSH Alter the Thiol Isomerase Activity of Purified $\alpha_{IIb}\beta_3$ in the Presence of Mn^{2+}. The effects of NO and GSH on the kinetic parameters of $\alpha_{IIb}\beta_3$ were determined in the presence of Mn^{2+}. $\alpha_{IIb}\beta_3$ was modified with a combination of the NO donor SNP and GSH as described in the Experimental Procedures section before addition to rdRNase A. The enzymatic activity of $\alpha_{IIb}\beta_3$ was measured as a function of increasing rdRNase A concentration. Nonlinear regression of the data also revealed a sigmoidal fit with $r^2 = 0.98$ (Figure 5A). A direct comparison of this kinetic behavior with that of the Mn^{2+} kinetics reveals that the enzymatic rate is modestly increased in the presence of NO and GSH particularly at the higher concentrations of substrate where a greater effect is observed. Linear transformation of the 10−90% range of these data into a Hill plot of log

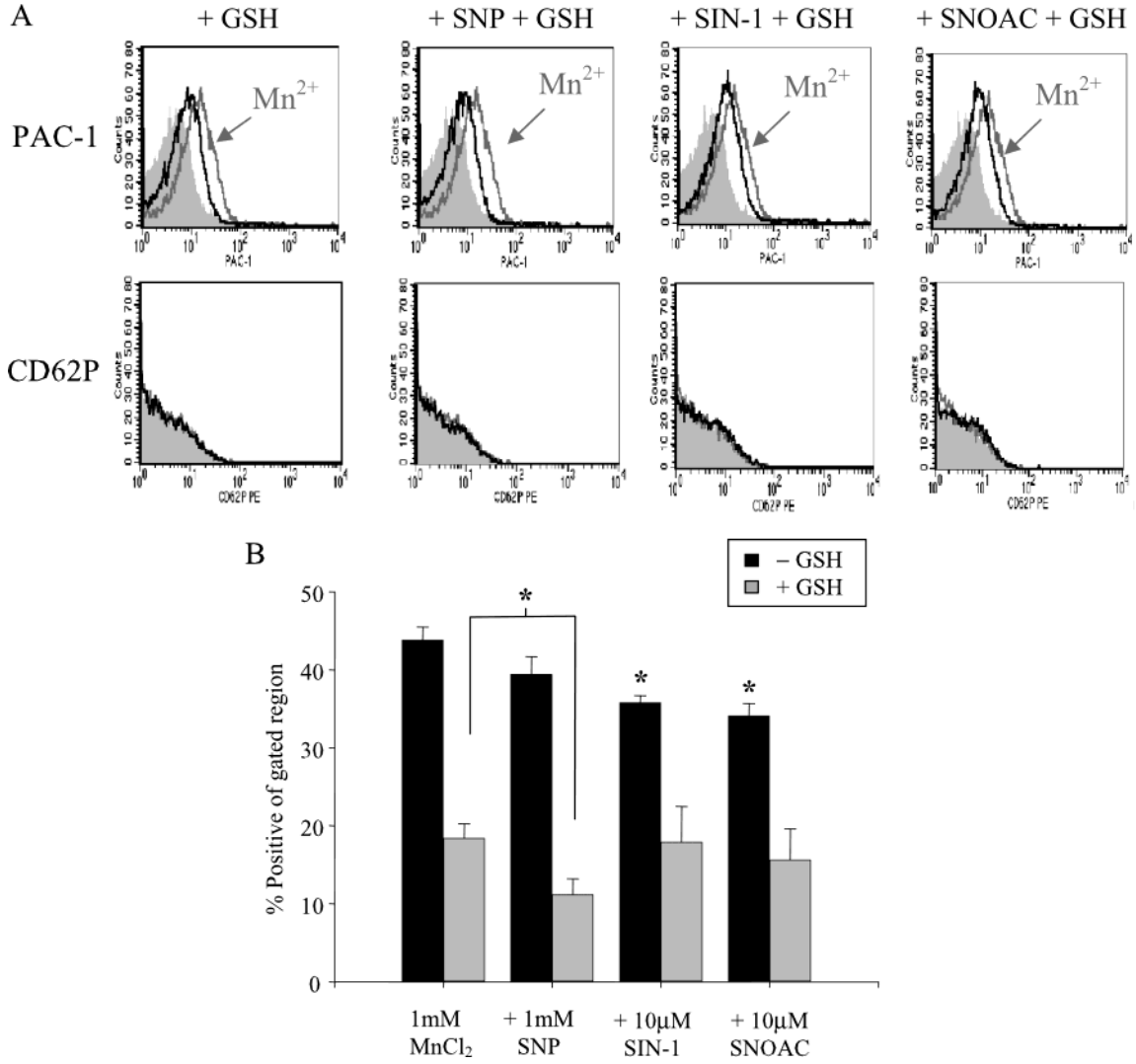

FIGURE 4: A combination of NO and GSH reverses PAC-1 binding to the Mn^{2+}-treated integrin. (A) Representative flow cytometric histograms of PAC-1 (upper panel) and CD62P (lower panel) binding to Mn^{2+}-treated (gray line) platelets. The addition of GSH (3 mM) alone or in combination with the NO donors SNP (1 mM), SIN-1 (10 μM), and SNOAC (10 μM) to Mn^{2+}-treated platelets decreases PAC-1 binding and has no effect on CD62P expression. The solid histograms (gray area) represent control platelets, and treatment is shown with a black line. (B) is the mean of three separate experiments ± SEM showing the NO donors SIN-1 and SNOAC significantly reverse the binding of PAC-1 ($p < 0.05$). GSH (3 mM, gray bar) also significantly reverses PAC-1 binding ($p < 0.001$). This effect is accentuated in the presence of SNP (1 mM) and was found to be significantly different from GSH alone ($p < 0.05$), indicating an additive effect of the two compounds.

[rdRNase] vs log $v_0/(V_{max} - v_0)$ also reveals positive cooperativity with an n_H of 1.9 and $r^2 = 0.92$ (Figure 5B). There was no change in $K_{0.5}$, which was found to have a value of 34 μM. It therefore appears that the thiol isomerase activity of $\alpha_{IIb}\beta_3$ is modulated in the presence of NO and GSH in such a way as to significantly alter the V_{max} without affecting the $K_{0.5}$.

DISCUSSION

The precise mechanism(s) by which $\alpha_{IIb}\beta_3$ is activated and deactivated is (are) still not fully understood but may involve structural cysteine residues (*18*) and the endogenous thiol isomerase activity of the integrin (*10*). Recently, it has been reported that activation of $\alpha_{IIb}\beta_3$ involves the modification of a redox site that has been assigned to the cysteine-rich core (CRR) of the β subunit where the EGF domains are present (*8*). Two to three free sulfhydryls were identified in a resting conformation of purified $\alpha_{IIb}\beta_3$ that increased to four to five in an active conformation of the purified protein. This suggests that upon activation of the receptor there is a reduction of one or two disulfide bonds within this core. Indeed, it is in the cysteine-rich EGF domains of this core that the CGXC motifs are present that predict the endogenous thiol isomerase activity of the integrin (*10*). Mutations in this domain can cause activation of both $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ (*19*). Furthermore, the epitope for ligand-induced binding site (LIBS) antibodies maps to this region (*9*). In addition, Yan and Smith (*20*) show that a specific cysteine within the CRR alters its disulfide partner during activation. A recent paper by Essex et al. demonstrates that an altered glutathione redox environment can potentiate platelet aggregation and also increase sulfhydryl labeling in the β subunit of $\alpha_{IIb}\beta_3$, suggesting that disulfide bond cleavage is involved in the activation of this integrin (*12*). Overall, these observations strongly suggest that thiol/disulfide exchange within the EGF domains of the β subunit plays a decisive role in the conformational changes that occur within $\alpha_{IIb}\beta_3$. We believe that these changes are most probably mediated by the

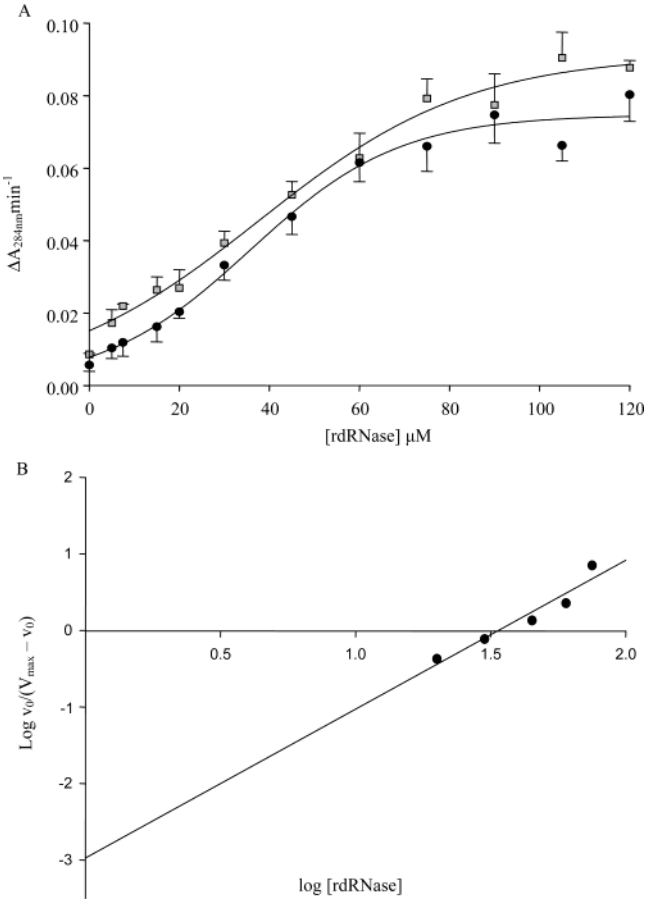

FIGURE 5: NO and GSH increase the V_{max} of the thiol isomerase activity of $\alpha_{IIb}\beta_3$ but not $K_{0.5}$. (A) shows the overlay of the Mn^{2+} saturation curves in the presence (shaded box) and absence (solid circle) of NO and GSH. The activity was measured using increasing concentrations of rdRNase (0−120 μM). $\alpha_{IIb}\beta_3$ was incubated with SNP (10 μM) and GSH (30 μM) for 5 min at room temperature. Excess NO and GSH were removed by centrifugation through a micro-bio-spin column, and the assay was performed after a 48 h incubation with 1.5 μM $\alpha_{IIb}\beta_3$. Nonlinear regression reveals a sigmoidal regression curve with $r^2 = 0.98$. Data shown are the mean of five experiments ± SEM. (B) Data were transformed into a Hill plot of log [rdRNase] vs log $v_0/(V_{max} - v_0)$. The Hill coefficient $n_H = 1.9$, indicating positive cooperativity, and $K_{0.5} = 34$ μM, with $r^2 = 0.92$.

endogenous thiol isomerase activity of the integrin. Thus modification of cysteine residues that constitute alterations in the functional properties of the receptor must be considered.

Interaction of platelets with a potent agonist such as thrombin results in both maximal activation of the platelet and conformational changes in $\alpha_{IIb}\beta_3$ leading to an increased affinity/avidity for ligands (*21*). The binding of ligands to integrins is without exception divalent cation dependent. For many integrins, ligand binding is stimulated by Mn^{2+} as was first shown for $\alpha_5\beta_1$ (*22*). Mn^{2+} cations are widely used to simulate integrin activation in intact cells as integrin-specific changes can be achieved without the confounding effects of generalized cellular activation (*23*). Furthermore, Mn^{2+} increases the ligand association rate of $\alpha_{IIb}\beta_3$ but does not induce the conformational changes equivalent to full activation (*17*). In purified preparations of $\alpha_{IIb}\beta_3$, we have previously shown that the thiol isomerase activity is optimal in the presence of the divalent cation chelator EDTA. In this study, we demonstrate that Mn^{2+} can also stimulate the endogenous thiol isomerase activity. Kinetic analysis revealed the allosteric nature of the enzymatic activity of this integrin in the presence of Mn^{2+}, based on the sigmoidicity of the kinetic curve. The binding of a regulatory compound, in this instance, Mn^{2+}, can therefore modulate the activity and conformation of the enzyme, leading to a rapid stimulation of the thiol isomerase activity of $\alpha_{IIb}\beta_3$. The protein displays positive cooperativity in the presence of Mn^{2+} as its n_H is greater than 1, indicating increased catalytic activity at a given concentration of available substrate. These results contrast with the kinetic analysis of the thiol isomerase activity in the presence of EDTA, which displays typical Michaelis−Menten kinetics (*10*).

Our results also establish that Mn^{2+} can specifically modulate $\alpha_{IIb}\beta_3$ conformation independent of platelet secretion in intact platelets. The magnitude of the PAC-1 expression achieved with Mn^{2+} is less than that obtained with the potent platelet agonist thrombin but is highly integrin-specific. Incubation of platelets with Mn^{2+} does not result in secretion, as seen with activation by thrombin, and so the level of CD62P expression in the Mn^{2+}-treated platelets remained at basal levels. Thus, Mn^{2+} simultaneously and specifically stimulates thiol isomerase activity in purified integrin and PAC-1 binding in intact platelets. This result is strongly suggestive of a physiological role for the integrin enzymatic activity and also suggests a molecular mechanism for this divalent cation-induced activation.

It is well established that thiols readily react with activated NO intermediates to form *S*-nitrosothiols (*24*) and that these are more abundant in plasma compared to reactive free $NO^{\bullet}$ (*25*). Furthermore, these adducts appear to be more stable than $NO^{\bullet}$ itself (*26*). GSH interacts with $NO^{\bullet}$ to form the stable adduct *S*-nitrosoglutathione (GSNO) (*27*) and has several pharmacological activities which include a protective effect on cells exposed to oxidants (*28*) and inhibition of platelet aggregation (*29*). A combination of NO and GSH can reverse an activated conformational state of purified $\alpha_{IIb}\beta_3$ (*8*). Our data on intact platelets correlate with these findings. We show that the addition of a combination of GSH and the NO donor SNP to the Mn^{2+}-treated platelets caused a subtle but significantly greater reversal in PAC-1 binding than either the addition of the NO donor or GSH alone. Several NO donors were used in order to ensure the specificity of the observed NO effect. It should be noted that the NO-donating capabilities of these compounds have slightly different properties given that SIN-1 donates both NO and superoxide (*30*), and SNOAC is an *S*-nitrosothiol, a stable NO donor (*31*).

Examination of the thiol isomerase activity of purified $\alpha_{IIb}\beta_3$ modified by the redox agents NO and GSH in the presence of Mn^{2+} also revealed sigmoidal kinetics. The n_H was also greater than 1, indicating positive cooperativity. There was an overall shifting of the curve to the left, indicating that the NO and GSH act as positive modulators of the enzymatic activity with high specificity. Interestingly, there is a significant increase in the V_{max} in the presence of the redox agents but no change in the $K_{0.5}$. Therefore, there is a consistent acceleration of the activity of the enzyme in the presence of NO and GSH.

Modification of cysteine residues with both NO and GSH is well-known to alter protein function. The concept of redox regulation as a dynamic signaling system in both mammalian

and bacterial cells has been emerging over the past number of years. Under physiological conditions, NO is known to modify single cysteine thiols of proteins with amazing specificity, and such alterations are dictated by a consensus motif whereby one or more cysteine residues are flanked by basic and/or acidic residues (*32*). A broad range of proteins have been identified where S-nitrosylation of a specific cysteine residue alters the functions of the protein, including hemoglobin (*33*), caspase (*34*), NFκB (*35*), Ras (*36*), and the ryanodine receptor (*37*). Moreover, factors influencing S-nitrosylation include the protein conformation and/or the presence of metal ions as determined in the studies of hemoglobin and the ryanodine receptor. Since $\alpha_{IIb}\beta_3$ is a dynamic molecule that undergoes conformational changes associated with the displacement of cations and also contains consensus nitrosylation motifs in the β subunit, it is plausible that NO can directly modify cysteine residues within this molecule. Our data suggest a causal link between these two phenomena and that NO specifically transforms the conformational state of activated $\alpha_{IIb}\beta_3$.

Addition of exogenous millimolar GSH to platelets in in vitro assays inhibits platelet aggregation with a corresponding reduction in TxB_2 production (*38*). In a manner similar to NO, GSH is known to readily form adducts with protein thiols in a process termed S-glutathionylation. Such modifications have been shown to alter the function of proteins, and S-glutathionylation of the active site cysteine residue of creatine kinase (*15*) and glyceraldehyde-3-phosphate dehydrogenase (*39*) resulted in the inactivation of both enzymes.

Allosteric enzymes are oligomers in which the subunits interact cooperatively; i.e., changes in the structure of one subunit may be translated into structural changes in adjacent subunits, an effect that is usually mediated by noncovalent interactions at the subunit−subunit interface. The integrin $\alpha_{IIb}\beta_3$ is a heterodimeric protein and upon platelet activation with potent agonists such as thrombin forms clusters on the platelet surface (*40, 41*). A potential substrate for the thiol isomerase activity of one $\alpha_{IIb}\beta_3$ oligomer could therefore be an adjacent $\alpha_{IIb}\beta_3$ that could ultimately lead to the clustering of this receptor. We show that Mn^{2+} causes $\alpha_{IIb}\beta_3$ to undergo conformational changes so that it is in an active-like state and it also allosterically modulates its endogenous thiol isomerase activity. A combination of NO and GSH accelerates this activity, increasing its V_{max}, and simultaneously causes the activated integrin on intact platelets to revert toward its resting state. This acceleration of the enzymatic activity of the enzyme is most pronounced at higher substrate concentrations. Therefore, probable modification of cysteine residues in $\alpha_{IIb}\beta_3$ by nitrosylation and/or glutathionylation increases the thiol isomerase activity with a concomitant reversal of an active-like conformation of this integrin in the intact platelet. These results suggest that the ligand-competent conformation of $\alpha_{IIb}\beta_3$ can be modulated by redox conditions in a process mediated by the endogenous thiol isomerase activity of this integrin, thus suggesting a plausible functional role for this activity in integrin activation. Further studies will be required to confirm the modification of cysteine residues in $\alpha_{IIb}\beta_3$. However, our results suggest that there may be significant potential for redox modulators to be used as pharmacological tools in regulating ligand-competent conformations in integrins.

NOTE ADDED AFTER PRINT PUBLICATION

The word Integrin was spelled incorrectly in the version of this article published on the Web 12/19/03 (ASAP) and in the January 20, 2004, issue (Vol. 43, No. 2, pp 473−480). The correct electronic version was published 1/30/04, and an Addition and Correction appears in the February 24, 2004, issue (Vol. 43, No. 7).

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