Conformational Regulation of the $\alpha_4\beta_1$-integrin Affinity by Reducing Agents: “Inside-out” Signaling is Independent and Additive to Reduction-Regulated Integrin Activation

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Summary
The α4β1 (VLA-4, CD49d/CD29) integrin is an adhesion receptor involved in the interaction of lymphocytes, dendritic cells and stem cells with extracellular matrix and endothelial cells. This and other integrins have the ability to regulate their affinity for ligands through a process termed “inside–out” signaling that affects cell adhesion avidity. Several mechanisms are known to regulate integrin affinity and conformation: conformation changes induced by separation of the C-tails, divalent ions and reducing agents. Recently, we described a fluorescent LDV-containing small molecule, which was used to monitor VLA-4 affinity changes on live cells. Using the same molecule, we also developed a FRET based assay to probe the ”switchblade-like” opening of VLA-4 upon activation. Here we have investigated the effect of reducing agents on the affinity and conformational state of the VLA-4 integrin simultaneously with cell activation initiated by “inside–out” signaling through G protein-coupled receptors or Mn2+ on live cells in “real-time”. We found that reducing agents (DTT and DMPS) induced multiple states of high affinity of VLA-4, where the affinity change was accompanied by an extension of the integrin molecule. Bacitracin, an inhibitor of reductive function of the plasma membrane diminished the effect of DTT but had no effect on the “inside-out” signaling. Based on this result and differences in the kinetics of integrin activation we conclude that conformational activation of VLA-4 by “inside-out” signaling is independent of and additive to the reduction-regulated integrin activation.
Introduction

The \( \alpha_4\beta_1 \) (VLA-4\(^1\), CD49d/CD29) integrin is a heterodimeric protein, and a member of the family of adhesion receptors, which is broadly expressed on lymphocytes, dendritic cells (1), and stem cells (2). VLA-4 has a flexible molecular structure that allows initial capture, tethering, rolling, and firm attachment of cells using the same counterstructure (3;4). These properties appear to result from regulation of affinity and conformation by “inside-out” signaling (5;6). While the precise molecular mechanism of the integrin conformational activation is unknown, significant understanding of this mechanism has been achieved in the last several years.

One model involves the “mechano-conformational” regulation of integrin affinity and conformation. It is based on the idea that the separation of the intracellular \( \alpha \) and \( \beta \) subunit tails may initiate “a piston-like or scissor-like motion” of the transmembrane domains (7). This motion results in a large conformational rearrangement of the integrin, accompanied by a “switchblade-like” opening of the molecule (8) and its conformational activation (5;6;9;10). This “tail separation model” is supported by the experiments by Lu et al., and Takagi at al. (11;12), in which unclasping of the link between C-terminal parts of the integrin subunits results in the conformational activation of the molecule. A direct demonstration of the spatial separation of the LFA-1 integrin tails by fluorescence resonance energy transfer (FRET) has been recently published (10). The tail separation might be induced by binding of adaptor proteins such as talin (a common adaptor protein that binds to the beta subunits) (13) or paxillin (an \( \alpha_4 \)-specific adaptor, whose binding is regulated by the phosphorylation of the integrin (14-16)).

Another model suggests that integrin conformation is regulated by the reduction of the disulfide bonds, and possibly involves protein disulfide isomerase (PDI) (17-19). PDI regulates disulfide exchange and conformationally induced shedding of L-selectin (20). Moreover,
dithiothreitol (DTT) and other reducing agents elevate integrin mediated cell adhesion avidity (21-23), and non-penetrating blockers of the free sulphydryls groups inhibit integrin mediated adhesion (22;24). An inhibitor of the reductive function of the plasma membrane, bacitracin, and anti-PDI mAb caused an inhibition of ligand binding to the β3-integrin (19).

A number of mutational studies have shown that disruption of disulfides in the integrin β3-subunit results in constitutively active integrins (Cys5, Cys435, Cys560, Cys598, Cys663, Cys687) (25-28). Truncated (aa 1-469) β3 - subunits lacking the Cys-rich domain form heterodimers that bind fibrinogen with high affinity (29). In addition, all 56 cysteines in the integrin beta subunits were found to be well conserved through evolution (see Fig. 1 in (30)). Thus, there is good evidence that reduction or disruption of the disulfide bonds could be an important mechanism in the regulation of the integrin dependent cell adhesion.

Recently, we have developed a new approach for monitoring the relationship between VLA-4 affinity, cell avidity and molecular conformation. Using a VLA-4 specific fluorescent probe, based on an LDV containing compound, we were able to monitor changes in integrin affinity and conformation in “real-time” on live cells in response to cell activation by “inside-out” signaling (31;32). We have also demonstrated a strong correlation between VLA-4 affinity and cell adhesion avidity (33).

The goal of this present report was to investigate the role of reducing agents in regulating integrin conformation and affinity. We found that reducing agents induced multiple affinity states of VLA-4, and the affinity changes were accompanied by an extension of VLA-4 detected using FRET. An inhibitor of reductive function of the plasma membrane, bacitracin, diminished the effect of reducing agents and had no effect on the “inside-out” signaling generated using G protein-coupled receptors. Based on these results and differences in the kinetics of integrin
activation we concluded that the activation of VLA-4 by “inside-out” signaling is independent of activation by reducing agents.

**Experimental Procedures**

**Materials.** The VLA-4 specific ligand (31-33) 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-proyl-L-alanyl-L-alanyl-L-lysine (LDV containing small molecule), and its FITC-conjugated analog (LDV-FITC) were synthesized at Commonwealth Biotechnologies, Inc. (Richmond, VA). Octadecyl rhodamine B chloride (R18) was from Molecular Probes Eugene, OR). All restriction enzymes were purchased from New England BioLab (Beverly, MA). All other reagents were from Sigma (St. Louis, MO).

**Cell Lines and Transfectant Construct.** The human monoblastoid cell line U937 was purchased from ATCC (Rockville, MD). Site-directed mutants of formyl peptide receptor (FPR) in U937 cells were prepared as described (34). High expressors were selected using the MoFlo Flow Cytometer, Cytomation, Inc., (Fort Collins, CO). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in RPMI 1640 (supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, pH 7.4, and 10% heat inactivated fetal bovine serum), then harvested and resuspended in 1 ml of HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl₂, and 30 mM HEPES, pH 7.4) containing 0.1 % HSA and stored on ice. The buffer was depleted of lipopolysaccharide by affinity chromatography over polymyxin B sepharose (Detoxigel, Pierce Scientific, Rockford, IL). Cells were counted using the Coulter Multisizer/Z2 analyzer (Beckman Coulter, Inc., Miami, FL). For experiments, cells were suspended with the same HEPES buffer at 1 X 10⁶ cells/ml and warmed to 37°C. The expression of VLA-4 was measured with fluorescent mAbs and quantified by comparison with a standard
curve generated with Quantum Simply Cellular microspheres (Bangs Laboratories, Inc., Fishers, IN) stained in parallel with the same mAb. This produces an estimate of the total mAb-binding sites/cell. Typically, we find 40,000 – 60,000 VLA-4 sites per U937 cell.

**Kinetic Analysis of Binding and Dissociation.** Kinetic analysis of the binding and dissociation of the LDV-FITC containing small molecule was described previously (31). Briefly, U937 cells (1 X 10^6 cells/ml) were preincubated in HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl_2, and 30 mM HEPES, pH 7.4) containing 0.1 % HSA at different conditions for 10-40 min at 37°C: divalent cations (Mn^{2+}, Ca^{2+}), DTT (up to 3 mM), or DMPS (up to 50 mM). Flow cytometric data were acquired for up to 1000 s at 37°C while the samples were stirred continuously at 300 rpm with a 5 x 2 mm magnetic stir bar (Bel-Art Products, Pequannock, NJ). Samples were analyzed for 30-120 s to establish a baseline. The fluorescent ligand was added and acquisition was re-established, creating a 5-10 s gap in the time course. For “real-time” activation experiments U937 cells were preincubated with 4 nM LDV-FITC containing small molecule for 15 min at 37°C. Then, data were acquired for 30-120 s to establish a baseline and DTT (3 mM), fMLFF (100 nM), or ATP (1 µM) was added. Acquisition was re-established, and data were acquired continuously for up to 1000 s. The concentration of the LDV-FITC containing small molecule chosen for the experiments (4 nM) is below the dissociation constant (Kd) for the binding to the resting (low affinity) VLA-4 (Kd ~ 12 nM), and above the Kd for the physiologically activated receptor (high affinity) (Kd ~ 1-2 nM) (31). Therefore, the transition from the low affinity to the high affinity receptor state leads to the increased binding of the probe (from ~ 25 % to ~ 70-80 %. of receptor occupancy respectively), which is detected as an increase in the mean channel fluorescence (MCF). For dissociation kinetic measurements, cell samples were preincubated with the fluorescent ligand (4-10 nM), treated with excess unlabeled LDV
containing small molecule (2 µM) and the dissociation of the fluorescent molecule was followed. The resulting data were converted to mean channel fluorescence versus time using FCSQuery software developed by Bruce Edwards.

**Cell Pretreatment with Bacitracin.** U937 cells were preincubated on ice for 1.5 h with 1 mM bacitracin. Prior to the experiment 4 nM LDV-FITC was added and cells were incubated at 37°C for an additional 15 min. Data were acquired with the flow cytometer for 30 sec to establish a baseline. Then DTT (3 mM), fMLFF (100 nM), or ATP (1 µM) was added. For dissociation experiments, cells were preincubated with bacitracin and LDV-FITC as described above and treated with excess unlabeled LDV containing small molecule (2 µM). Then the dissociation of the fluorescent molecule was followed.

**FRET Detection of the Integrin Conformational Activation.** The fluorescence resonance energy transfer (FRET) assay used a peptide donor (LDV-FITC, which specifically binds to the α4-integrin headgroup) and octadecyl rhodamine B acceptors incorporated into the plasma membrane previously described in detail in (32). Briefly, U937 cells were preincubated with 50-100 nM LDV-FITC to saturate low affinity sites of the integrin in HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl2 and 30 mM HEPES, pH 7.4) containing 0.1 % HSA supplemented with 1 mM Mn²⁺, 1 mM Ca²⁺, 1-3 mM DTT, or a combination of the reagents for up to 50 min at 37 °C. Next, samples were incubated with different concentrations of R18 (up to 20 µM) for 1 min. Then donor intensities (FL1) were measured using a Becton-Dickinson FACScan flow cytometer at 37°C.

The quenching curves generated using the following procedure characterize the distance of closest approach between the integrin headgroup and the surface lipid membrane as was shown previously (32). For “real-time” FRET experiments, U937 cells were stably transfected with the
wild type or the non-desensitizing mutant (ΔST) of the formyl peptide receptor (35;36). The U937 cells were preincubated with 50-100 nM LDV-FITC in HHB buffer containing 1.5 mM CaCl₂ and 1 mM MgCl₂ at 37°C. Samples were analyzed for 60-120 s to establish a baseline, and then saturating R18 (10 µM final) was added to yield maximal quenching. 1 min after R18 was added, fMLFF peptide (0.1 µM), ATP (1 µM), or DTT (3 mM) was added. FACS acquisition was re-established, after a 5-10 s gap. The cells were also tested using low (3-5 nM) concentration of LDV-FITC to determine the affinity change as described above.

**Statistical Analysis.** Curve fits and statistics were performed using GraphPad Prism (San Diego, CA). Mean values are presented in Table I and Figures. Each experiment was repeated three times. The experimental curves represent the mean of two independent runs. Standard error of the mean was calculated using GraphPad Prism (GraphPad Software Inc.).
Results

Reducing agents generate multiple affinity states of the VLA-4 integrin as detected using fluorescent ligand—Multiple affinity states of VLA-4 have been detected using the LDV-FITC containing molecule in “real-time” in response to activation by divalent cations, activating mAb, or “inside–out” signaling in response to the stimulation of CXCR2, CXCR4, FPR, IgE, and IL-5 receptors (31). The affinity of the LDV-FITC probe for the integrin varies in parallel with the affinity of a native ligand (VCAM-1). Cell adhesion avidity was found to be strongly dependent on the affinity of the integrin (33;37). Here, we used the same LDV-FITC containing molecule to probe the affinity of VLA-4 on a surface of U937 cells treated with different concentrations of DTT and DMPS. DMPS is known to be a membrane-impermeant reducing agent due to the presence of a charged acidic group. Fig. 1A shows a typical binding and dissociation experiment in which the LDV-FITC molecule was added to a cell suspension after 30 s of stirring. An excess of unlabeled competitor was added 3 min later. By fitting dissociation kinetics to double exponential curves we extracted rate constants corresponding to states of different affinity (Table I). In all experiments, with the exception of untreated cells, a combination of high and low affinity state receptors was detected (Fig. 1A, B and C). For untreated cells only a single exponential fit was needed, and a $k_{off} \sim 0.06-0.1 \text{ s}^{-1}$ was obtained. This off-rate corresponds to the resting receptor state (31). In addition, a resting state was detected on cells treated with low concentrations of reducing agents (300 µM DTT and 1 to 20 mM DMPS) (Table I).

Changes in VLA-4 affinity were strongly dose dependent and fits required at least two dissociation rates. Under the strongest reducing conditions (3mM DTT), the dissociation could be fit with two rates (0.014/sec and 0.002/sec), or with three rates, resembling the resting, intermediate, and high affinity states. For consistency, all the data were fit with three fixed rates,
higher concentrations of DTT and DMPS or longer incubation times resulting in a larger fraction of high affinity receptors (Table I). The progression of quantitatively similar states led to the idea that a sequential reduction of each of the disulfide bonds generates a distinctive conformation of the molecule (Fig. 1D).

**Kinetics of the affinity changes induced by Mn$^{2+}$ and DTT in “real-time”** — Next, “real-time” activation was used to measure the kinetics of the VLA-4 affinity change. U937 cells were preincubated with LDV-FITC and treated with DTT alone or in combination with 1 mM Mn$^{2+}$ (Fig. 2). Mn$^{2+}$ is used to induce a higher affinity state of VLA-4 with a distinctive extended conformation (31;32). The effect of Mn$^{2+}$ was stable and irreversible for more than 1000 s. The addition of DTT induced a slow and gradual increase in LDV-FITC binding. This was completely different from a rapid activation induced by Mn$^{2+}$ (Fig. 2A). Next, when the two stimuli were added together biphasic binding kinetics were observed. A rapid binding phase (60-120 s) that resembles “Mn$^{2+}$ alone” curve was followed by a slow gradual signal increase similar to the curve “DTT alone” (Fig. 2A). Analysis of the dissociation kinetics (Fig. 2B) confirmed that DTT, when added together with Mn$^{2+}$, created higher VLA-4 affinity than Mn$^{2+}$ alone (slower dissociation rate corresponds to a state of higher affinity (31)).

Exposure of disulfides to solvent is a factor that regulates the rate of disulfide reduction by reducing agents. Therefore, we investigated whether a conformational change of the integrin molecule induced by cations affects the response to DTT. We subtracted the curve corresponding to the activation by Mn$^{2+}$ alone (Fig. 2A, filled circles) from the curve “DTT & Mn$^{2+}$ (Fig. 2A, open circles)”. The resulting curve is plotted in Fig. 2C (open circles) together with DTT alone curve from Fig. 2A (triangles). We found that the slope of the curve “DTT & Mn$^{2+}$ - Mn$^{2+}$” was approximately two times larger than for DTT alone. Thus, the rate of DTT-induced activation of
Regulation of the Integrin Affinity and Conformation by Reducing Agents

integron was higher for the extended conformation induced by Mn$^{2+}$. This suggests that the conformational change in VLA-4 induced by ions facilitates a subsequent change in the affinity induced by DTT. Presumably, this could be achieved by exposing disulfides, which are less accessible to DTT in the resting conformation.

Next, cells were preincubated with Mn$^{2+}$ in the presence or absence of DTT (Fig. 2D) to test whether the addition of DTT can generate a higher affinity state than induced by Mn$^{2+}$ alone. In our previous experiments the dissociation constant ($K_d$) for the binding of LDV-FITC in 1 mM Mn$^{2+}$ (in absence of other divalent ions) was ~ 0.1-0.3 nM and $k_{off} \sim 0.0005-0.0007$ s$^{-1}$ (33). Fig. 2D shows that $k_{off}$ was at least 5 times slower for DTT treated cells ($k_{off} \sim 0.0001$ s$^{-1}$). This value corresponds to $K_d \sim 20-60$ pM. Thus, addition of DTT induced a higher affinity state than for divalent cations only. This suggests that the mechanism of the integrin activation by reducing agents is independent and additive to the one induced by ions.

Kinetics of the affinity changes induced through “inside-out” signaling differ from the activation by DTT — Next, to determine if DTT affects integrin activation through “inside-out” signaling, cells were treated with DTT alone or in combination with activation using two GPCR ligands: fMLFF (ligand for FPR (Fig. 3)), and ATP (ligand for P2Y receptors (Supplemental Fig. 1)). Whereas U937 cells were transfected with the FPR (34;36), a family of P2Y receptors (purinergic receptors) is constitutively expressed on U937 cells (38-40). These activation experiments were performed in “real-time”.

Treatment of the cells with DTT induced a gradual increase in LDV-FITC binding (Fig. 3A). In these experiments we used a higher concentration of DTT (3 mM) in comparison to the Mn$^{2+}$ experiments (1 mM). Therefore, the slope of the curve for the DTT treated cells was ~ 3 times higher (compare Fig. 2C, slope ~ 0.07, and Fig. 3C, slope ~ 0.23, or supplemental Fig. 1C,
slope 0.20). As shown previously, cell activation through GPCRs induces rapid and reversible change in the integrin affinity (FPR, Fig. 2, and P2Y receptor, supplemental Fig. 1) in ref. (31). Fig. 3B shows the presence of two affinity states in the experiments where DTT, or DTT and fMLFF were added (low affinity, k_{off} \sim 0.04-0.06 \text{ s}^{-1}, corresponding to the resting receptor state, and high affinity k_{off} \sim 0.004 \text{ s}^{-1}). A larger fraction of high affinity receptors was detected when DTT and fMLFF were added together when compared with DTT or fMLFF alone (compare values in Fig. 3B next to dissociation curves). When cells were activated through FPR (Fig. 3B, filled circles) only one dissociation component (k_{off} \sim 0.04 \text{ s}^{-1}) was detected. This result is consistent with rapid desensitization of the wild-type FPR (31). Thus, the kinetics of the DTT induced LDV-FITC binding, that reflect the kinetics of the VLA-4 affinity changes (31), are dramatically different from the activation by “inside-out” through GPCR.

Fig. 3C shows the curve corresponding to “fMLFF alone” (Fig. 3A, filled circles) subtracted from the curve “DTT & fMLFF” (open circles), and plotted together with “DTT alone” curve. This curve (Fig. 3C, open circles) has two slopes; a higher slope starting from 70 s to 240 s (~0.39) that was interpreted as a faster rate of disulfide reduction caused by the conformational change induced through “inside-out” signaling, and a lower slope (~ 0.23) has exactly the same value as by DTT alone (compare Fig. 3C open circles after 240 s, and filled triangles). We hypothesize that the “inside-out” signaling generated by FPR activation results in a conformational rearrangement of the integrin, and this leads to the exposure of integrin disulfides, as in the case of activation by Mn^{2+} (Fig. 3D). Thus, a conformational change facilitates an activation of the integrin by DTT. After desensitization and termination of receptor signaling (after ~ 240 s) integrins returned to their resting affinity (31), and conformational (32) state. As a result disulfide bonds became less accessible to the reducing agent. After ~240 s the
slope of the line (Fig. 3C, open circles) became ~ 0.23. This value corresponds to the resting non-extended molecule (Fig. 3C, triangles, Fig. 3D). Essentially the same behavior of integrins with a faster kinetics was observed when VLA-4 was activated by ATP through P2Y receptors. P2Y2, and P2Y6 are GPCRs nucleotides receptors constitutively expressed on U937 cells (38-40) (Supplemental Fig. 1).

It is worth noting that the ratio between the two slopes for “DTT & fMLFF – fMLFF” and “DTT alone” (Fig. 3C; 0.39/0.2 ~ 2, from 70 s to 240 s) was approximately the same as for Mn$^{2+}$ activation (Fig. 2C; 0.16/0.07 ~ 2). We detected ~2-fold difference in the rate of reduction between the folded and the extended conformation. Thus, the extension of integrins induced by ions and by “inside-out” signaling result in similary facilitated reduction, but having different kinetics: long and persistent for the case of ions, short and reversible for the case of GPCRs activation.

**Bacitracin diminishes the effect of DTT on integrin activation, but has no effect on the response induced by “inside-out” signaling**— Recently, it has been proposed that protein disulfide isomerase (PDI) present on the cell surface participates in the regulation of integrin-dependent adhesion (17-19;24), and could be a part of “outside-in” and/or “inside-out” signaling pathways (17). To clarify the role of PDI in the “inside-out” activation of the integrin, we used bacitracin, an inhibitor of reductive function of the plasma membrane (20;41). Preincubation of U937 cells with 1 mM bacitracin significantly diminished the rate of the DTT-induced activation of the VLA-4 (compare slopes on a Fig. 4A). On the contrary, no statistically significant inhibition of the “inside-out” integrin activation through FPR or P2Y receptors was detected (Fig. 4 B,C). Bacitracin had no effect on the integrin affinity of resting cells; the dissociation rate was similar for treated and non-treated cells  ($k_{off} \sim 0.04 \text{ s}^{-1}$, Fig. 4D). Nonspecific binding of
fluorescent substances present in the bacitracin solution results in different baselines for treated and non-treated cells (Fig. 4D). Thus, the reductive capacity of the plasma membrane had no effect on VLA-4 activation by intracellular signaling. This result is more consistent with the “mechano-conformational” theory of integrin regulation than with involvement of the reduction-related mechanisms in the “inside-out” integrin activation.

Fluorescence Resonance Energy Transfer based detection of integrin extension induced by DTT—A FRET based method was used to detect molecular extension of integrins (32). The LDV-FITC small molecule was used as a fluorescence donor and R18 incorporated into the membrane as an acceptor. U937 cells were treated with different concentrations of DTT, and divalent ions (Fig. 5). As we have shown previously for ions and “inside-out” signaling (32), activation of VLA-4 by DTT results in decreased FRET efficiency. This was interpreted as an increase in the distance of closest approach between the integrin ligand-binding site and the surface of the membrane. An estimate of the distance between the headgroup of the VLA-4 and the cell membrane for 1 mM Mn$^{2+}$ + 3 mM DTT was ~ 60-90 Å (32). This estimate was based on a calibration of acceptor surface densities for the resting receptor in 1 mM Ca$^{2+}$ and was defined to be 0 Å separation distance. Thus, the activation of VLA-4 using DTT results in the extension of the integrin, in which the headpiece is moving away from the membrane (Fig. 5C).

Kinetics of DTT-induced extension, detected using FRET, coincides with the kinetics of affinity changes — Finally, for a “real-time” FRET based assay (32) cells were preincubated with a large excess of the LDV-FITC small molecule. Next, the fluorescent signal was quenched using R18. Then, cells were activated through different GPCRs or DTT. Addition of DTT induced slow and gradual unquenching of the fluorescence signal (Fig. 6B, triangles). However, the “inside-out” signaling promoted an instant unquenching that reflects rapid extension of the
integrin molecule as was shown previously (32). Thus, the kinetics of the conformational extension of VLA-4, detected using FRET, were different between “inside-out” signaling and activation by DTT as shown for the affinity change.
Discussion

Multiple affinity state of the VLA-4 integrin – In circulating lymphocytes, VLA-4 has the potential to exhibit multiple affinity states that mediate tethering, rolling and arrest on its endothelial ligand, vascular cell adhesion molecule-1 (VCAM-1) (42-44). We have used the LDV-FITC small molecule as a model ligand that reports the affinity state of VLA-4 under different activating conditions (31). The fluorescent probe was based on the structure of BIO1211, a highly specific $\alpha_4\beta_1$ integrin inhibitor developed by Biogen Inc (43;45). Previously, we found that LDV-FITC can be used to determine the affinity of the natural VLA-4 ligand VCAM-1, and that changes in the integrin binding affinity to VCAM-1 coincided with changes in a cell adhesion avidity (33) and molecular conformation (32). For activation by DTT most of the variation in the affinity of the probe arose from the changes in the dissociation rate, rather than association rate, as shown for activation by divalent ions, activating antibodies, and “inside-out” signaling (31;33). The difference in dissociation constant values for BIO1211 was also governed almost exclusively by dissociation rates (43). This situation is probably typical for the type of receptors in which the conformation of the ligand-binding pocket determines the residence time of the ligand. For integrins the change in the ligand affinity and the residence time could be sufficient to slow down cell rolling, and to result in cell arrest and firm adhesion of the leukocytes. The reported difference between the highest and the resting affinity state of VLA-4 is more than 2 orders of magnitude (31;33). This concept is additionally supported by the result that stable cell aggregates could be formed between VLA-4 and VCAM-1 expressing cells connected only by one or two bonds at the states of different affinity (46).

In contrast, when integrins were activated by reducing agents as shown here, several distinctive affinity states of VLA-4 were detected in the cell population at the same time (Table
I). The kinetics of activation by DTT and the changes in VLA-4 affinity were slow, and were dependent on time and concentration. Longer incubation times at higher concentrations of reducing agents resulted in a larger fraction of high affinity VLA-4. These data differed from integrin activation using divalent ions, where usually only one affinity state was detected. Fits to the dissociation data require one exponential curve (see Fig. 1B and C in (33), (43)). The VLA-4 activating mechanism may explain the above difference: for quickly diffusing divalent ions at high concentrations (usually 1-3 mM) equilibrium is reached rapidly resulting in a similar state for all the receptors. For the reductive activation involving disulfide-exchange reactions, and, possibly enzymatic reactions catalyzed by PDI (17-19;24), several discrete states of integrin activation occur, presumably, by reducing different numbers of disulfide bonds in different molecules. Fig. 1D shows a hypothetical mechanism that relates the number of reduced disulfides to the conformational state of the VLA-4.

Previously, the rapid interconversion between the resting state and the physiologically activated state was demonstrated using the LDV-FITC small molecule (see Fig.5 in (31)). However, in this case, only two affinity states ($k_{off1} \sim 0.06$ s$^{-1}$, and $k_{off2} \sim 0.01$ s$^{-1}$) were detected. The affinity state generated using the highest concentration of DTT was at least 10 times higher (Table I), than the physiologically activated receptor. Thus, the magnitude of the affinity changes after reductive activation of VLA-4 was significantly different from the one generated through “inside-out” signaling.

Kinetics of the affinity changes and cell activation – Because it has been recognized that integrin affinity can be regulated by a mechanism related to disulfide bond reduction (17;19;21;24), our goal was to determine whether affinity regulation by DTT could occur on a proper time frame to be physiologically relevant. We found that the kinetics of integrin
activation induced by high concentrations of reducing agents (up to 3 mM of DTT, up to 50 mM of DMPS) was very slow in comparison to activation through GPCRs (Figs. 2-4, and supplemental Fig.1). Moreover, knowing that reducing agents generate populations of different affinity receptors, our data support the idea that two different mechanisms result in the state of higher integrin affinity. For “inside-out” signaling it could be separation of C-tails of the α and β subunits, resulting in a large conformational change (7;10-12). For reducing agents it could be an enzymatic mechanism that involves PDI-catalyzed disulfide exchange reactions (17-19;21;24). To further test this hypothesis we used bacitracin, a drug that is known to inhibit the reductive function of the membrane (20;41).

“Inside-out” signaling and reducing agents – Several ideas connecting “inside-out” signaling and integrin activation led us to investigate the effect of the bacitracin on the integrin activation induced by DTT simultaneously with the signaling though GPCRs. These include a “DTT-sensitive regulatory element” (21), as well as requirements for PDI enzymatic activity for integrin activation (19), adhesion (24), and aggregation (17). We showed that bacitracin reduced the rate of DTT induced conformational activation of the integrin (Fig. 4A), but had no inhibitory effect on integrin activation by “inside-out” signaling (Fig. 4B and C). In fact, bacitracin caused slightly slowed desensitization of the LDV-FITC signal in the case of fMLFF stimulation (Fig. 4B, open circles between 100 s and 300 s). These data suggest that regulation of integrins by reducing agents was essentially independent of “inside-out” signaling whereas the GPCR or Mn²⁺ induced conformational change increased the rate of integrin activation by reducing agents (Figs. 2, 3 and Supplemental Fig.1).

Integrins, three or more independent activating mechanisms – Integrin conformational change and activation can be achieved under different conditions: divalent ions, reducing agents,
“inside-out” signaling, as well as mutations of extracellular domains, C-terminal tails, or disulfide disruption (11;23;25;26;43). Several opposing mechanisms may contribute to integrin conformational change with divalent ions. The central metal-ion-dependent adhesion site (MIDAS) has two geometries, and is regulated by two other polar sites: one adjacent to the MIDAS site and the other, a ligand-induced metal binding site (47). In this scenario, the Ca\(^{2+}/\)Mn\(^{2+}\) competition is critical for the regulation of the ion-mediated cell adhesion. However, another report shows that the Ras-like small GTPase Rap1 is necessary for the activation of integrins by Mn\(^{2+}\) or activating antibodies (48). In this scenario, intracellular signaling could be involved in the regulation of integrin dependent cell adhesion in response to Mn\(^{2+}\) or TS2/16 mAb. Our data showed that changes in VLA-4 affinity could be detected after incubating cells on ice with Mn\(^{2+}\) or activating antibodies, suggesting that an ion/antibody-induced conformational change of the molecule rather than intracellular signaling was sufficient for increased affinity (31). Moreover, the VLA-4 affinity state induced by Mn\(^{2+}\) or TS2/16 was several orders higher than “physiologically-activated” state induced by the “inside-out” signal. Thus, the affinity/conformational state induced by Mn\(^{2+}\) or activating mAbs was “non-physiological” although it may have reflected the continuum of states available to the flexible molecule under physiological conditions. It was impossible to achieve the high affinity similar to the Mn\(^{2+}\) or mAbs induced state via only “inside-out” signaling through GPCRs (31-33).

There is a similar dichotomy for the relevance of conformation and signaling to disulfide reduction. One line of research showed that activation of integrin-dependent cell adhesion by DTT or other reducing agents requires cell signaling, cytoskeleton, and PDI activation (17-19;21;22;24). Since PDI participates in the regulation of L-selectin shedding (20), it is tempting to propose a PDI related mechanism as a general regulatory feature of both selectins and
integrins, the two main classes of adhesion molecules. Another report suggests that the redox site within the extracellular domain of the integrin molecule functions as “on/off switch that regulates ligand binding affinity” (49;50). The reduction of disulfides could “mechanically” lead to global conformational changes and the opening of the ligand binding sites.

Our data show that the affinity state of VLA-4 generated using membrane permeable and impermeable reducing agents was much higher than the state induced by “inside-out” signaling. The kinetics of DTT-induced VLA-4 activation was slow in comparison to GPCR stimulation. Moreover, the presence of large amounts of reducing agent during cell activation had no significant effect on “inside-out” activation. Bacitracin, had no effect on integrin activation via GPCR signaling, but significantly reduced DTT induced activation. These data suggest that integrin activation by “inside-out” signaling is therefore not associated with disulfide reduction (5;9;12)). The exposure and reduction of disulfides within VLA-4 upon activation (49) is more likely to be a result of conformational rearrangement than the cause of it. For reducing agents, the slow reduction of disulfides was facilitated by the conformational change and the associated extension of VLA-4 that was detected using FRET. In our view, the states produced by disulfide reduction are therefore not physiological, although the affinities observed may represent a continuum of affinities accessible to the flexible VLA-4 molecule and encompass those induced by Mn$^{2+}$, activating antibodies, and molecular stretching (see below).

_Regulation of VLA-4 affinity and conformation provide a “catch-bond” mechanism –_ Previously we showed a progressive increase in VLA-4 affinity, a decrease in ligand dissociation rate, and an increase in distance of closest approach of the ligand binding site to the membrane as the integrin was activated by divalent ions or GPCRs (31;32). In this report we used a mechanistically different approach to activate integrins – activation by reducing agents. We
found that a progressive decrease in the LDV-FITC dissociation rate was accompanied by extension of the integrin molecule detected using endpoint and “real-time” FRET based assays (Fig. 1, Fig. 5, and Fig. 6). A strong correlation between the affinity states of VLA-4 and the degree of the molecular extension support the idea that the conformational change involving VLA-4 extension also affects ligand binding affinity (Fig. 7). These data provide a novel mechanism accounting for an adhesion “catch bond” (51): a mechanical stretching of a flexible integrin molecule during cell rolling or under shear that would induce a high affinity conformation of the integrin, and result in higher cellular adhesive avidity.
References


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Footnotes

1 Abbreviations:

DMPS, 2,3-dimercapto-1-propane-sulfonic acid – membrane membrane-impermeable reducing agent
DTT, dithiothreitol
fMLFF, N-formyl-L-methionyl-L-leucyl-L-phenylalanyl-L-phenylalanine
FPR, formyl peptide receptor 1
FRET, fluorescence resonance energy transfer
GPCR, G-protein coupled receptor
HSA, human serum albumin
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LDV containing small molecule, 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine
LDV-FITC containing small molecule, 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine-FITC
mAb, monoclonal antibody
MCF, mean channel fluorescence
PDI, protein disulfide isomerase
VCAM-1, vascular cell adhesion molecule 1, CD106
VLA-4, very late antigen 4, CD49d/CD29, α4β1 integrin
Table I

Summary of dissociation rate constants for U937 cells treated with different concentrations of DTT and DMPS.

<table>
<thead>
<tr>
<th>Affinity states</th>
<th>Dissociation rate, $k_{off}$, s$^{-1}$</th>
<th>Fraction of VLA-4 receptors in this affinity state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.06$^a$</td>
<td>0.01$^b$</td>
</tr>
<tr>
<td>Cell treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.97</td>
<td>0.03</td>
</tr>
<tr>
<td>300 µM DTT</td>
<td>0.61</td>
<td>0.14</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.28</td>
<td>0.19</td>
</tr>
<tr>
<td>3 mM DTT</td>
<td>0.12</td>
<td>0.30</td>
</tr>
<tr>
<td>1 mM DMPS$^c$</td>
<td>0.78</td>
<td>0.09</td>
</tr>
<tr>
<td>20 mM DMPS</td>
<td>0.45</td>
<td>0.42</td>
</tr>
<tr>
<td>50 mM DMPS</td>
<td>0.20</td>
<td>0.57</td>
</tr>
</tbody>
</table>

The data were fit to the equation $MCF = Span_1 * e^{-k_1 \cdot T} + Span_2 * e^{-k_2 \cdot T} + Span_3 * e^{-k_3 \cdot T} + Plateau$; where $MCF$ (mean channel fluorescence) represents total binding, $Plateau$ is non-specific fluorescence, $T$ is time, $k_n$ is dissociation rate constant, $Span = Span_1 + Span_2 + Span_3$ is equal to the difference between binding at time zero and $Plateau$. Dissociation rate constants were fixed at $k_1 = 0.06$ s$^{-1}$, $k_2 = 0.01$ s$^{-1}$, and $k_3 = 0.002$ s$^{-1}$. The $Span$ value was assigned to be equal 1 ($Span_1 + Span_2 + Span_3 = 1$). Next, a fraction corresponding to $Span_1$, $Span_2$ and $Span_3$ was calculated. These values, corresponding to a fraction of VLA-4 receptors in each affinity state,
are shown in Table I. For comparison, a two-component fit is shown for 3 mM DTT (dissociation rate is shown in parenthesis).

\(^a\)This LDV-FITC dissociation rate corresponds to the low affinity (resting) state of VLA-4 (31;33).

\(^b\)This LDV-FITC dissociation rate corresponds to the physiologically activated affinity state (31).

\(^c\)Because of the difference in redox potentials DMPS was used in a higher concentration than DTT.
Figure legends

Fig. 1. **Binding and dissociation of the LDV-FITC – containing small molecule on U937 cells.** Experiments were conducted as described under “Experimental Procedures”. A, LDV-FITC binding and dissociation on U937 cells plotted as mean channel fluorescence versus time, after sequential additions of fluorescent (4 nM) and nonfluorescent (2 µM) LDV-containing small molecule (arrows). U937 cells were pretreated in HEPES buffer with 1 mM of DTT for 10 min at 37°C (open circles), or vehicle (untreated, filled circles). Values of mean channel fluorescence corresponding to the cell autofluorescence and non-specific binding of the LDV-FITC-containing small molecule indicated by dashed arrows. B, LDV-FITC dissociation plotted as mean channel fluorescence versus time. U937 cells were preincubated for 40 min at 37°C with indicated concentrations of DTT in presence of 4 nM LDV-FITC. Next, nonfluorescent (2 µM) LDV-containing small molecule was added to induce probe dissociation (arrow). Curves were fitted to a one phase exponential curve (untreated, crosses), or two phase exponential curve (all others). Calculated off rate constants are presented in Table I. C, the same experiment as shown in panel B, but DMPS was used instead of DTT. D, a cartoon showing a hypothetical mechanism implying that sequential reduction of the disulfides (-S-S- → -SH + HS-) results in a change of the conformation/affinity of the integrin.

Fig. 2. **Response kinetics of LDV-FITC binding to U937 cells following stimulation by Mn^{2+} and DTT.** A, U937 cells were preincubated with 4 nM LDV-FITC in HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂ and 30 mM HEPES, pH 7.4) containing 0.1 % HSA for 5-10 min at 37°C. Next, DTT (1 mM, arrow # 2, filled triangles), Mn^{2+} (1 mM, arrow # 2, filled circles), or sequentially DTT (1 mM, arrow # 1, open circles), and
Mn$^{2+}$ (1 mM, arrow # 2, open circles) were added. B, dissociation of LDV-FITC initiated by addition of nonfluorescent LDV (2 µM, arrow) for the cells treated as described in A. Dissociation rate constants shown on the graph were obtained by fitting data to single exponential curves. C, the data corresponding to the Mn$^{2+}$ experiment (A, filled circles) was subtracted from the data of cells treated with DTT and Mn$^{2+}$ (A, open circles), and plotted on the same panel with “DTT alone” from panel A (C, filled triangles). The baseline value 140 (shown on panel A by dashed line) was subtracted from “DTT alone” data. The slope of the curve “DTT & Mn$^{2+}$ - Mn$^{2+}$” remains constant over time. The slope of the control curve “DTT alone” on Fig. 2 (slope ~ 0.07) (C, filled triangles) is ~ 1/3 the slope in Fig. 3 (slope ~ 0.2) (C, filled triangles) because of the lower DTT concentration used (3 mM for the experiment shown in Fig. 3, and 1 mM in Fig. 2). D, LDV-FITC small molecule dissociation from U937 cells treated with 1 mM Mn$^{2+}$ in HEPES buffer in the absence of other divalent cations (Ca$^{2+}$ and Mg$^{2+}$) in the presence or absence of DTT (3 mM for 40 min at 37°C). Dissociation rate constants shown on the graph were obtained by fitting the data to single exponential curves. Binding was plotted as mean channel fluorescence versus time.

Fig. 3. **Response kinetics of LDV-FITC binding to U937 cells following stimulation by fMLFF and DTT.** A, U937 cells transfected with wild type formyl peptide receptor were preincubated with 4 nM LDV-FITC for 10 min at 37°C. Next, DTT (3 mM, arrow # 2, filled triangles), fMLFF (0.1 µM, arrow # 2, filled circles), or sequentially DTT (3 mM, arrow # 1, open circles), and fMLFF (0.1 µM, arrow # 2, open circles) were added. B, dissociation of LDV-FITC initiated by addition of nonfluorescent LDV (2 µM, arrow) for the cells treated as described in A for 500 s. Dissociation rate constants shown on the graph were obtained by fitting
data to single (fMLFF only, filled circles), or double exponential curves. Numbers in parentheses represent a fraction of VLA-4 receptors in each affinity state calculated as described in the legend for Table I. C, the data corresponding to the fMLFF experiment (A, filled circles) were subtracted from the data corresponding to the cells treated with DTT and fMLFF (A, open circles). The result is plotted on the same panel with “DTT alone” from panel A (C, filled triangles). Baseline value 220 (shown on a panel A by dashed line) was subtracted from “DTT alone” data. The different slopes of the curve “DTT & fMLFF- fMLFF” correspond to different rates of integrin activation by DTT. Binding was plotted as mean channel fluorescence versus time. D, a cartoon showing a hypothetical mechanism that links rapid and reversible “inside-out” signaling with the exposure of the disulfide bonds and reductive activation of the integrin.

**Fig. 4. The effect of bacitracin on integrin activation by DTT or “inside-out” signaling detected using LDV-FITC.** A, U937 cells transfected with wild type formyl peptide receptor were preincubated on ice for 1.5 hour without or with 1 mM Bacitracin (Bac). Then, cells were incubated at 37°C with 4 nM LDV-FITC. A, cells were activated with 3 mM DTT. B, cells were activated with 0.1 µM fMLFF. C, cells were activated with 1 µM ATP (P2Y nucleotide receptors constitutively expressed on U937 cells (38-40)). D, dissociation of the LDV-FITC-containing small molecule from the cells preincubated with or without Bac (as described in A). The difference in the baseline for Bac treated and non-treated cells was due to nonspecific binding of fluorescent substances present in the Bac solution (compare plateaus of the dissociation curves on a panel D). Dissociation rate constant shown on the graph was obtained by fitting data to single exponential curve. Binding was plotted as mean channel fluorescence versus time.
Fig. 5. **Energy transfer on U937 cells between LDV-FITC donor and octadecylrhodamine (R18) acceptor.** Measurements were made as described in “Experimental Procedures” (32). A, fluorescence intensity plotted as a function of R18 concentration under three conditions: 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$, and 1 mM Mn$^{2+}$ + 3 mM DTT for 40 min at 37°C. Data are plotted as specific fluorescence of LDV-FITC (fluorescence signal corresponding to the sample blocked with 2 µM nonfluorescent LDV was subtracted, therefore the Y-axes are labeled as “ΔMCF”). Inset, data from Fig. 5A replotted as relative quantum yield versus acceptors/$R_0^2$. Curves represent a simulation of energy transfer as a function of donor distance of closest approach expressed in term of $R_0$ according to Wolber and Hudson model (52). The surface densities were estimated based on the lateral FRET using fluorescein C18/ rhodamine C18 on U937 cells (see Fig. 3 in (32)). Because the Wolber and Hudson model is only valid for acceptor densities of <0.5 acceptors/$R_0^2$ the analysis of the data in Fig. 5A is truncated. The data shown in the inset represents the analysis of the data shown in Fig 5A in the box as limited by the FRET model. B, Quenching data are plotted for two DTT concentrations and untreated cells in a buffer containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ (incubation for 40 min at 37°C). Inset, data from Fig. 5B replotted as for the inset in Fig. 5A. C, schematic of FRET methodology. The left panel shows an integrin heterodimer in the inactive conformation (bent). Upon activation the integrin assumes an extended (upright) conformation. Changes in FRET efficiency between LDV-FITC donor bound to the headpiece of the molecule and octadecylrhodamine acceptor (R18) incorporated into the membrane were used to estimate the distance of the closest approach of the donor and acceptor molecules (32).
Fig. 6. "Real-time" FRET experiments with integrin activation by “inside-out” signaling and reducing agent. U937 cells were stably transfected with the nondesensitizing mutant of formyl peptide receptor (ΔST) (36) and preincubated at 37°C with 100 nM LDV-FITC to saturate low affinity sites in buffer containing 1 mM Ca²⁺ and 1 mM Mg²⁺. Next, LDV-FITC fluorescence was quenched after addition of 10 µM octadecyl rhodamine R18 (arrow). Cells were then activated by addition of 0.1 µM of fMLFF, or 3 mM DTT. A, data are plotted as mean channel fluorescence versus time for two conditions: quenched and then activated by fMLFF (open circles), and quenched only (baseline, filled triangles). B, comparison of the integrin conformational activation by fMLFF and 3 mM DTT in “real-time”. Data plotted by subtracting the baseline data from activated cell data; therefore the Y-axis is labeled as “ΔMCF”. Because the formyl peptide receptor mutant ΔST does not desensitize, the VLA-4 remains in a state of the constant affinity (31).

Fig. 7. Correlation between the affinity states of VLA-4 and the degree of the molecular extension determined using FRET. Separation distance ($r_c$) plotted as a fraction $R_0$ of versus logarithm of the dissociation rate ($k_{off}$, s⁻¹) of LDV-FITC small molecule for five different affinity states. For fluorescein – rhodamine pair $R_0 = 55\text{Å}$. Putative VLA-4 conformations depicted as a cartoon. Data from several previous publications (31-33) and present report were used.

Supplemental Fig. 1. Response kinetics of the LDV-FITC small molecule binding to cells following stimulation by ATP and DTT. A, U937 cells constitutively expressing P2Y nucleotide receptor were preincubated with 4 nM LDV-FITC for 10 min at 37°C. Next, DTT (3
mM, filled triangles), ATP (1 µM, open circles), or sequentially 3 mM DTT, and 1 µM ATP, (filled circles) were added. B, dissociation of the LDV-FITC small molecule initiated by addition of nonfluorescent LDV (2 µM) for the cells treated as described in A. Dissociation rate constants shown on the graph were obtained by fitting data to double exponential curves. C, the curve corresponding to the ATP experiment (A, open circles) was subtracted from the curve corresponding to the cells treated with DTT and ATP (A, filled circles), and plotted in the same panel with “DTT alone” from panel A (C, filled triangles). The baseline value 190 (shown on a panel A by dashed line) was subtracted from “DTT alone” curve. The two different slopes of the curve “DTT & ATP- ATP” reflect different rates of the integrin activation by DTT. Binding was plotted as mean channel fluorescence versus time.
Figure 1

A: Regulation of integrin affinity and conformation by reducing agents. The graph shows the effect of different DTT concentrations (0, 1 mM, 3 mM) on cell autofluorescence over time. Untreated cells are indicated by the black line, with LDV block at 4 nM. The graph also includes 2 µM LDV-FITC and non-specific binding.

B: Effect of different DTT concentrations on cell autofluorescence. The graph shows the time course of MCF (mean cell fluorescence) over time for untreated cells and cells treated with 1 mM, 3 mM DTT. Untreated cells are indicated by the black line, with LDV block at 2 µM.

C: Effect of DMPS on cell autofluorescence. The graph shows the time course of MCF over time for untreated cells and cells treated with 1 mM, 20 mM, 50 mM DMPS. Untreated cells are indicated by the black line, with LDV block at 2 µM.

D: Time course of binding for untreated cells and cells treated with 1 mM, 20 mM, 50 mM DMPS. The graph shows the change in MCF over time for untreated cells and cells treated with different concentrations of DMPS, indicating the regulation of integrin affinity and conformation.
Figure 2

A) 

B) 

C) 

D)
Figure 3

A) 

B) 

C) 

D) 

Rapid and reversible “inside-out” signaling
Figure 4

A

Bac & DTT

DTT

Slope ~ 0.22

Slope ~ 0.30

B

fMLFF

Bac & fMLFF

C

1 mM Bac & 100 nM ATP

100 nM ATP

D

LDV

LDV-FITC dissociation from untreated cells

k_{off} \sim 0.04 \text{ s}^{-1}
Figure 5

A

B

C

Regulation of the Integrin Affinity and Conformation by Reducing Agents
Figure 6

(A) Regulation of the integrin affinity and conformation by reducing agents. (B) Changes in MCF (Baseline subtracted).

- R18 only
- R18 & fMLFF
- fMLFF
- DTT

Delta MCF (Baseline subtracted)

Time (s)
Regulation of the Integrin Affinity and Conformation by Reducing Agents

Figure 7

![Graph showing the effect of reducing agents on Integrin affinity and conformation.](image)

- 1 mM Mn^{2+} + 3 mM DTT
- 1 mM Mn^{2+} + 1 mM Ca^{2+}
- 1 mM Mn^{2+} + 1 mM Ca^{2+}

- Increase in VLA-4 affinity
- Log (k_{off})

- Resting state
- Activated by "inside-out" signaling

- r_{c} (fraction of 55Å)

- r_{c} (fraction of 55Å)

- Actively increased conformation

- Coefficient of determination: r^2 = 0.7
- p < 0.002
Supplemental Figure 1