Integrin Activation Involves a Conformational Change in the α1 Helix of the β Subunit A-domain

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The ligand-binding region of integrin β subunits contains a von Willebrand factor type A-domain: an α/β “Rossmann” fold containing a metal ion-dependent adhesion site (MIDAS) on its top face. Although there is evidence to suggest that the β-domain undergoes changes in tertiary structure during receptor activation, the identity of the secondary structure elements that change position is unknown. The mAb 12G10 recognizes a unique cation-regulated epitope on the β1 α-domain, induction of which parallels the activation state of the integrin (i.e. competency for ligand recognition). The ability of Mn2+ and Mg2+ to stimulate 12G10 binding is abrogated by mutation of the MIDAS motif, demonstrating that the MIDAS is a Mn2+/Mg2+ binding site and that occupancy of this site induces conformational changes in the A-domain. The cation-regulated region of the 12G10 epitope maps to Arg154/Arg155 in the α1 helix. Our results demonstrate that the α1 helix undergoes conformational alterations during integrin activation and suggest that Mn2+ acts as a potent activator of β1 integrins because it can promote a shift in the position of this helix. The mechanism of β subunit A-domain activation appears to be distinct from that of the A-domains found in some integrin α subunits.

Integrins are α/β heterodimeric transmembrane receptors that have widespread essential functions in development, tissue organization, and the immune system (1). Integrins recognize a variety of extracellular matrix and cell-surface ligands; however, ligand recognition is frequently not constitutive but is instead under strict cellular control by “inside-out” signaling. Acquisition of the active state has also been shown to require divalent cations. For β1 integrins, ligand binding is promoted by Mg2+ or Mn2+ but only weakly by Ca2+ (2). A well known but unexplained property of Mn2+ is its ability to mimic the process of inside-out signaling to strongly up-regulate integrin function (3, 4).

The molecular basis of integrin-ligand interactions has been greatly elucidated by the recent x-ray crystal structure of α5β3 (5). The ligand binding “head” of the integrin is seen to contain a seven-bladed β-propeller fold in the α subunit and a von Willebrand factor type A-domain in the β subunit (βA-domain) (6). Cation-binding sites are present on the lower face of the β-propeller domain and the upper face of the βA-domain (5). The key regions involved in ligand recognition are loops on the upper surface of the β-propeller and the upper face of the βA-domain, which contains a metal ion-dependent adhesion site (MIDAS) (5–7). Nevertheless, as the crystal structure only provides a “snapshot” of one integrin conformation, attention is now focused on understanding the conformational changes that occur during the transition from the inactive to active state (8). These changes are thought to include shape shifting in the βA-domain (4, 7).

The A-domain contains a central hydrophobic β sheet encircled by seven α helices (α1–α7) (5). Some α subunits also contain an A-domain and a key feature of the activation of these domains has been shown to be a large movement of the α7 helix (9). Here we investigate conformational changes in the βA-domain using the anti-β1 mAb 12G10, which recognizes a cation- and ligand-induced epitope (10, 11). We show that movements in the α1 helix of the βA-domain parallel changes in the activation state of the integrin. Our results provide insights into the mechanisms of Mn2+ and Ca2+-induced shape changes in the β1 subunit, and therefore into the opposing roles played by these divalent ions in regulating integrin function. Our findings also imply that the mechanism of βA-domain activation is different to that of αα-domains.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—Rat mAbs 16 and 13 recognizing the human α5 and β3 subunits, respectively, were gifts from Dr. K. Yamada (NIDCR, National Institutes of Health, Bethesda, MD). Mouse anti-human α5 mAb SNAK52 and mouse anti-human β3 mAb 12G10 were produced as described (10, 12). Mouse anti-human mAb TS2/16 was a gift from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Mouse anti-human mAbs 4B4 and P4C10 were purchased from Beckman Coulter (High Wycombe, UK) and Invitrogen (Paisley, Scotland, UK), respectively. All mAbs were used as purified IgG except P4C10 (as asacites).

Expression Vector Construction and Mutagenesis—C-Terminally truncated human α5 and β3 constructs encoding α5 residues 1–613 and β3 residues 1–455 fused to the Fc region of human IgG1; CHO, Chinese hamster ovary.

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Activation Mechanism of Integrins

19801

10% fetal calf serum, 2 mM glutamine, and 1% nonessential amino acids (growth medium). Cells were detached using 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS, and plated overnight into six-well culture plates (Costar). 1 μg of wild-type or mutant β1(1–455)-Fc, and 1 μg of α5(1–613)-Fc DNA was used to transfect the cells using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. After 4 days, medium was harvested by centrifugation at 1000 × g for 5 min.

For comparison of wild-type trα5β1-Fc with trα5β1-Fc containing the D130A or R154R/AS mutations in β1, 75-cm² subconfluent CHOL7616 cells were transfected with 5 μg of wild-type or mutant β1(1–455)-Fc and 5 μg of α5(1–613)-Fc DNA as described above. After 4 days, culture supernatants were harvested at 1000 × g for 5 min. Wild-type or mutant heterodimers were purified using Protein A-Sepharose essentially as described previously (13).

Proteins—A recombinant fragment of fibronectin containing type III repeats 6–10 (III6–10) was produced and purified as previously described (15). III6–10 and mAb 12B10 were biotinylated as before (11, 15), except that sulfo-LC-NHS biotin (Perbio, Chester, UK) was used in place of sulfo-NHS biotin.

Effect of Divalent Cations on Binding of mAb 12G10—Purified integrin was dialyzed to approximately 1 μg/ml in Dulbecco’s PBS and added to the wells of a halfera-area enzyme immunoassay/radio immunoassay plate (Costar, Corning Science Products, High Wycombe, UK, 25 μl/well) for 16 h at room temperature. Wells were blocked for 1–3 h with 200 μl of 5% (w/v) BSA, 150 mM NaCl, 0.05% (w/v) NaN3, 25 mM Tris-Cl, pH 7.4 (blocking buffer). Wells were then washed three times with 200 μl of 150 mM NaCl, 25 mM Tris-Cl, pH 7.4, containing 1 mg/ml BSA (buffer A). Buffer A was treated with Chelex beads (Bio-Rad, Hemel Hempstead, UK) to remove any small contaminating amounts of endogenous Ca2+ and Mg2+ ions. 12G10 (0.1 μg/ml) in buffer A with varying concentrations of Mn2+, Mg2+, or Ca2+ was added to the plate (50 μl/well). The plate was then incubated at 30 °C for 2 h. Unbound antibody was aspirated, and the wells washed three times with buffer A. Bound antibody was quantitated by addition of 1.500 dilution of ExtrAvidin® peroxidase-labeled conjugate (Sigma, Poole, UK) in buffer A followed by room temperature (50 μl/well). Wells were then washed four times with buffer A, and color was developed using 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (50 μl/well). Background binding to BSA was subtracted from all measurements. Measurements obtained were the mean ± S.D. of four replicate wells.

For comparison of the effects of divalent cations on 12G10 binding to wild-type trα5β1-Fc and the R154R/AS mutant, the assay was performed using 12G10 concentrations that gave approximately half-maximal antibody binding in 1 mM Mn2+ (0.1 μg/ml for wild-type trα5β1-Fc and 0.05 μg/ml for R154R/AS mutant). Binding was measured in 2 mM EDTA, Mn2+, Mg2+, or Ca2+. Measurements obtained were the mean ± S.D. ± S.D. of four replicate wells.

Effect of Divalent Cations on Binding of III6–10—Measurements of the binding of III6–10, to purified wild-type or mutant trα5β1-Fc was performed exactly as described for biotinylated 12G10 (see above), except that biotinylated III6–10 was incubated with integrin for 3 h at 30 °C. All assays were performed using a concentration of biotinylated III6–10 that gave approximately half-maximal ligand binding in 1 mM Mn2+ (0.1 μg/ml).

Sandwich ELISA for Epitope Expression—A 96-well plate (Costar half-area enzyme immunoassay/radio immunoassay) was coated with goat anti-human γ1 Fc (Jackson Immunochemicals, Strathe Scientific, Luton, UK) at a concentration of 2.6 μg/ml in Dulbecco’s PBS (50 μl/well) for 16 h. The coating solution was replaced with blocking buffer for 1 h. The blocking solution was removed, and cell culture supernatants were added (25 μl/well) for 1 h. All supernatants were assayed in triplicate, and supernatant from mock-transfected cells was used as a negative control. The plate was washed three times in buffer A containing 1 mM MnCl2 (buffer B; 200 μl/well), and anti-α5 or anti-β1 mAbs (10 μg/ml, or 1 μg/ml for SNAK52) were added (50 μl/well). The plate was incubated for 2 h and then washed three times in buffer B. Peroxidase-conjugated anti-rat or anti-mouse secondary antibodies (1:1000 dilution in buffer B; Jackson Immunochemicals) were added (50 μl/well) for 30 min. The plate washed four times in buffer B, and color was developed using 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (50 μl/well). All steps were performed at room temperature.

Each experiment shown is representative of at least three separate experiments.

FIG. 1. Effect of divalent cations on the binding of mAb 12G10 (A) and III6–10 fragment of fibronectin (B) to trα5β1-Fc. Binding of 12G10 or III6–10 was measured in the presence of varying concentrations of Mn2+, Mg2+, or Ca2+. For tests of specificity, the binding of biotinylated 12G10 to trα5β1-Fc could be inhibited >95% by a hundredfold excess of unlabelled 12G10; III6–10 binding to trα5β1-Fc could be inhibited >90% by the anti-α5 mAb 16 (data not shown).

β1 residues 1–455 fused to the hinge regions and Cα2 and Cα3 domains of human IgG1 were generated as previously described (13). To aid heterodimerization, the Cα3 domain of the α5 construct contained a “hole” mutation, whereas the Cα3 domain of the β1 construct carried a “knob” mutation as described (13, 14). Mutations in the A-domain of the β1 subunit were carried out using oligonucleotide-directed PCR mutagenesis, as described (13). Oligonucleotides were purchased from MWG Biotech (Milton Keynes, UK). The presence of the mutations was verified by DNA sequencing.

Transfection—Chinese hamster ovary cells L761h variant (13) were maintained in Dulbecco’s modified Eagle’s medium supplemented with...
TABLE I
Summary of mAb reactivity with β, A-domain mutants

| β, mutant | Anti-α, | Anti-β, |
|-----------|---------|---------|
| D130A     | +++     | +++     | +++     |
| N151A     | +++     | +++     | +++     |
| M153A     | +++     | +++     | +++     |
| R154A     | +++     | +++     | +++     |
| R155A     | +++     | +++     | +++     |
| I156A     | +++     | +++     | +++     |
| R154A/AS  | +++     | +++     | +++     |

CHO L761h cells were transfected with α,β(1–613)-Fc and wild-type or mutant β,β(1–455)-Fc. Cell culture supernatants were analyzed for reactivity with anti-α, and anti-β, mAbs by sandwich ELISA. The anti-α, mAbs recognize the β-propeller domain (12), and the anti-β, mAbs are all directed against the βA-domain (11, 21). ++++, reactivity >100% of wild-type integrin; +++, reactivity 50–100% of wild-type integrin; +, reactivity 20–50% of wild-type integrin; +/−, reactivity <20% of wild-type integrin. None of the mutations (except D130A) affected recognition of the IIIA10 fragment of fibronectin (data not shown).

RESULTS

Induction of the 12G10 Epitope on the β, A-domain Correlates with Competency for Ligand Binding—To investigate the mechanisms of integrin activation, we employed a recently described system for expression of recombinant soluble αβ1 (13). For these particular studies, we have used a truncated version of αβ1, α,β(1–613)β,β(1–455), fused to the Fc region of human IgG1γ1 (hereafter referred to as trαβ1-Fc). This heterodimer contains the ligand-binding head and thigh domains of the integrin (5) and has been shown to retain the properties of the full-length receptor (13). In contrast to previous mutagenesis-based analyses of integrin function, which have largely employed cell-expressed integrins, this system is ideal (a) because it permits the rapid analysis of the effects of mutations and (b) because the effects of mutations that normally preclude expression at the plasma membrane can be studied. 12G10 is a previously characterized activating mAb directed against the β, A-domain, whose binding to αβ1 is modulated by divalent cations and ligand (10, 11). The binding of 12G10 to trαβ1-Fc was promoted by Mn2+ and to a lesser degree by Mg2+, whereas Ca2+ was inhibitory (Fig. 1A). The effects of these cations on 12G10 binding closely paralleled their effects on ligand binding (Fig. 1B). Importantly, as for the native integrin, ligand binding is strongly activated by Mn2+ and more weakly by Mg2+, whereas Ca2+ is a very poor activator (16). These results show that the βA-domain undergoes conformational changes in response to cation binding (reported by modulation of the 12G10 epitope) that correspond with changes in the activation state of the integrin.

Mutation of the MIDAS Site in the βA-domain Leads to Loss of Induction of 12G10 Binding by Mn2+/Mg2+—The identity of the cation-binding site(s) involved in activation of β, integrins by Mn2+ and Mg2+ is unknown. The MIDAS is a strong candidate for this site, but this has been difficult to test experimentally because mutation of the MIDAS residues completely abrogates ligand recognition (although expression is unaffected; Refs. 17–19). In agreement with these previous studies, trαβ1-Fc with MIDAS mutations did not bind ligand under any cation conditions, even though such mutations (e.g. D130A) retained all the epitopes of conformation-sensitive α, and β, mAbs (Table I). Because mAb binding was retained, we tested the effect of the D130A mutation on the ability of divalent cations to regulate 12G10 binding to trαβ1-Fc (Fig. 2). The binding of 12G10 to the D130A mutant in the absence of divalent cations was similar to the wild-type integrin (comparing Fig. 1A with Fig. 2); however, the ability of Mn2+ and Mg2+ to stimulate 12G10 binding was totally lost in the MIDAS mutant. Interestingly, the inhibition of 12G10 binding by Ca2+ seen for the wild-type integrin was enhanced in the MIDAS mutant. Similar results were obtained with a “double” MIDAS mutation D130A/S132A (data not shown). Conversely, muta-

*2 A. P. Mould, J. A. Askari, S. Barton, and M. J. Humphries, manuscript in preparation.
not perturb the binding of other function-modulating mAbs. 

**Figure 3.** Effect of R154R/AS mutation on cation-modulated binding of 12G10 (A) and fibronectin fragment IIIe-149 (B) to trαβ-Fc. Binding of 12G10 or IIIe-149 to wild-type trαβ-Fc or trαβ-Fc with the mutation R154R/AS in β1 was measured in the presence of 2 mM EDTA (white bars), 2 mM Mn2+ (black bars), 2 mM Mg2+ (gray bars), or 2 mM Ca2+ (hatched bars). In A, 12G10 was used at concentration of 0.1 μg/ml for wild-type trαβ-Fc or 10 μg/ml for the R154R/AS mutant (conditions that gave approximately half-maximal 12G10 binding in the presence of 2 mM Mn2+). The control mutation M153A had no effect on the cation modulation of 12G10 binding (data not shown).

**Figure 4.** Comparison of conformational changes in α and β subunit A-domains. A model of the β1 A-domain was built using an alignment against the β1 A-domain crystal structure (5) in the program PROCK (version 3.5, Molecular Applications Group, Palo Alto, CA; Ref. 42). Modeling was carried out using an automated segment matching algorithm, followed by restrained energy minimization refinement under SEGMOND (43, 44). Representations of A-domains were produced using SETOR (45), using the Protein Data Bank entry 1JLM for the closed form of the α2 A-domain. β strands are shown in blue, α helices are shown in red, and cysteine bridges are in yellow. The divalent ion at the MIDAS site is depicted by a green sphere. In the model of the β1 A-domain, the positions of the α1, α2, and α7 helices are indicated by thin arrows, and the side chains of Arg154 and Arg155 are shown as green sticks. The metal ion at the MIDAS site of the βα A-domain (5) is omitted for the sake of clarity. The two A-domains are viewed from approximately the same orientation. In the transition from the closed (inactive) to the open (active) form of the α2 A-domain, there is a large downward shift of the α7 helix and a small inward movement of the α1 helix (thick arrows). In the transition from the inactive to active form of the β1 A-domain, the α1 helix changes position, whereas the α7 helix may not move. The α2 helix region contains the epitopes of activating and inhibitory anti-β1 mAbs (21).

**DISCUSSION**

Using the anti-β1 mAb 12G10 as a probe of βα-domain conformation, we have shown that: (i) the βα-domain undergoes shape changes that correlate with changes in the activation state of the integrin, (ii) occupancy of the MIDAS site in the βα-domain by Mn2+ or Mg2+ induces these changes, and (iii) βα-domain activation involves movement of the α1 helix. Taking these results together, we propose that the βα-domain can exist in at least two conformational states: an "active" conformation with the α1 helix in a position characterized by high 12G10 binding and an "inactive" conformation with the α1 helix in a different position, characterized by low 12G10 binding.

Movement of the α1 helix appears to form an essential part of the activation mechanism of the βα-domain because α1 movement closely parallels the activation state and a lack of α1 be predicted to be in sufficiently close proximity to Lys218 in the α2 helix for all three residues to contribute to the 12G10 epitope (23).

The above data suggest that Arg154 and Arg155 form part of the 12G10 epitope, but these residues do not contribute to other A-domain epitopes. Hence, do Arg154/Arg155 form the cation-regulated region of the 12G10 epitope? To test this proposal, we compared the effects of divalent cations on 12G10 binding to the R154R/AS mutant and wild-type trαβ-Fc. The results (Fig. 3A) showed that the abilities of Mn2+, Mg2+, and Ca2+ to modulate 12G10 binding were strongly attenuated by the R154R/AS mutation. The mutation did not affect the cation regulation of ligand binding (Fig. 3B) or of αα epitopes (Ref. 11; data not shown), suggesting that the mutation does not itself affect cation-induced conformational changes but rather that the ability of 12G10 to detect these changes is specifically compromised by the mutation. Therefore, the portion of the 12G10 epitope that is responsive to cation binding lies in the α1 helix, indicating that the position of this helix is different in the active and inactive states.

In further support of this proposal, 12G10 reacts only weakly with a chimeric β1 containing Asn151, Met153, Ile156 of human β1 in a backbone of chicken β1 (Ref. 21), in contrast to the other function-modulating mAbs, which show good reactivity. In chicken β1, Arg154 and Arg155 are both altered to Glu and Lys, respectively, whereas other residues in this region are unchanged (W. Puzon-McLaughlin, Y. Takada, A. P. Mould, and M. J. Humphries, unpublished observations).
movement (in the Ca^{2+}-occupied integrin) corresponds to low activity. Furthermore, the epitopes of function-blocking anti-chicken β₂ mAbs have been shown to include residues in the α₁ helix (24), and the epitopes of function-altering anti-human β₁ mAbs include residues in the α₂ helix, which lies adjacent to α₁ (5, 21). Based on previous analyses of the mode of action of regulatory anti-integrin mAbs (2, 25), it appears that they are likely to function allosterically by stabilizing the position of α₁ in either the active or inactive conformation. Additionally, it has been shown that mutation of residues in the α₁ helix can activate ligand binding (26).

Our data provide evidence that the MIDAS is primarily a Mn^{2+}/Mg^{2+} binding site and suggest an explanation for the opposing effects of Mn^{2+} and Ca^{2+} on β₁ integrin function. Mn^{2+} can induce a large shift in the equilibrium between active and inactive states because of its ability to promote α₁ helix movement upon binding to the MIDAS site. On the other hand, Ca^{2+} is unable to cause the same conformational change. It is likely that Ca^{2+} can occupy the MIDAS site because Ca^{2+} can support low affinity ligand binding to α₃β₁ and high affinity binding of activation-independent ligands to α₅β₁ (34). However, Ca^{2+} binding to sites other than the MIDAS appears to shift the equilibrium toward the inactive conformation (low 12G10 binding), as shown by the strong inhibition of 12G10 binding by Ca^{2+} in the D130A mutant.

Some integrins contain an A-domain in their α subunits (e.g. the β₂ family). These domains can exist in inactive (“closed”) or active (“open”) states dependent on movement of the C-terminal helix (α7). The open form can be induced in the presence of a ligand or pseudo-ligand, or by locking the position of α7 (9, 27–29). There appear to be some differences between the activation mechanism of αA-domains and the βA-domain. First, the nature of the metal ion at the MIDAS does not directly influence the equilibrium between inactive and active states in αA-domains (4, 28), whereas, based on data reported here for the βA-domain, the nature of the divalent ion can markedly affect this equilibrium. Second, in contrast to the βA-domain, there is no evidence for allosteric regulation of activity by mAbs to αA-domains whose epitopes include residues in the α₁ helix (30). Third, although in αA-domains the open form can be induced by mutation of residues that form a hydrophobic pocket surrounding α7 (31), mutation of the equivalent residues in β₁ does not alter integrin activity.² Fourth, the crystal structure of the β₃ A-Domain indicates that the α7 helix is unlikely to undergo large conformational movements (5). All these findings suggest that the βA-domain is regulated differently to the αA-domains in that movement of the α₁ helix (rather than α7) is a key feature of βA-domain activation. Nevertheless, comparison of the open and closed forms of αA-domains shows that there is an inward shift of the α₁ helix in the open form (9), and a similar movement could take place in the βA-Domain (Fig. 4).

In integrins that contain an A-domain in the α subunit, the βA-domain does not participate directly in ligand binding (4). Nevertheless, Mn^{2+} and mAbs to the βA-domain can strongly modulate the activity these integrins (3, 4). The epitopes of activating and inhibitory anti-β₂ mAbs have also been shown to contain residues in the α₁ helix of the β₂ A-Domain (4, 32, 33). Therefore, movement of the α₁ helix may also regulate the activation state of this class of integrin.

Is α₁ helix movement involved in the activation of integrins by inside-out signaling? It has been shown that the expression of the 12G10 epitope correlates with the activity of cell-surface β₁ integrins, whereas expression of other β₁ A-Domain epitopes is constitutive (35, 36). Because 12G10 differs from the other A-Domain mAbs in having part of its epitope in the α₁ helix, these data imply that inside-out signaling alters the position of α₁. Inside-out signaling may also cause a shift in the conformation of the α₁ helix in β₂ integrins. For α₁β₂ and α₅β₂, activating cytoplasmic domain mutations led to the induction of the α₂B4 epitope, which includes Arg^{122} in the α₁ helix of the β₂ A-Domain (4, 37). It has often been questioned whether Mn^{2+}-induced integrin activation accurately mimics physiologic activation. However, a common feature of both types of activation appears to be movement of the α₁ helix; hence, their molecular mechanisms may be very similar.

Finally, how might α₁ helix movement be important for activation? The top (MIDAS) face of the βA-domain interacts closely with the upper surface of the α subunit β-propeller domain (5). In particular, loops on the top face of the A-domain close to the α₁ helix (notably the α₂–α₃ loop) contact loops on the β-propeller domain that participate in ligand recognition. Hence, α₁ helix movement is likely to affect the α subunit/β subunit interface, potentially leading to changes in exposure of the ligand binding loops. In support of this hypothesis, we have shown that divalent cations affect the binding of inhibitory mAbs on the α subunit (11); the epitopes of these mAbs include residues in the same loops that are important for ligand recognition (12, 38, 39). In integrins with an A-domain in the α subunit, there is evidence that the MIDAS face of the βA-domain is in contact with the lower face of the αA-domain (40); hence, conformational changes in the βA-domain could affect the position of the α7 helix in the αA-domain and thereby alter the activation state of this domain.

In summary, we have shown that a conformational shift in the α₁ helix of the βA-domain is involved in the regulation of integrin activity. Integrins are important therapeutic targets in many inflammatory and vascular disorders (41), and our findings suggest a novel way in which highly specific regulators of integrin activity could be developed. A more complete understanding of the activation mechanism will require crystallization of an integrin in both active and inactive states.

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Note Added in Proof—After this manuscript was accepted for publication, the crystal structure of integrin α₅β₂ in complex with an RGD ligand was reported (Xiong, K.-P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S., and Arnauot, M. A. (2002) Science 296, 151–155). The partial conformational change between the unliganded (5) and liganded structures is an inward movement of the α₁ helix in the βA-domain. This conformational change appears to be causally linked to occupancy of the MIDAS site by a Mn^{2+} ion in the liganded structure.

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