Different cation binding to the I domains of α1 and α2 integrins: implication of the binding site structure

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Abstract In the present work, we studied the interactions of recombinant α1 and α2 integrin I domains with cations Tb^{3+}, Mn^{2+}, Mg^{2+} and Ca^{2+}. We observed that α1 and α2 I domains bind these cations with significantly different characteristics. The binding of Mg^{2+} by the α1 I domain was accompanied by significant changes of tryptophan fluorescence which could be interpreted as a conformational change. Comparison of the α1 integrin I domain structure obtained by comparative modeling with a known structure of the α2 integrin I domain shows distinct differences in the metal ion binding sites which could explain the differences in cation binding.

Key words: Integrin; I domain; Cation binding; Terbium fluorescence; Comparative modeling

1. Introduction

The integrins are a family of plasma membrane proteins that transduce bidirectional signals between the cytoplasm and the extracellular matrix or other cells [1]. Seven of the integrin α subunits described to date, α1, α2, αL, αX, αD, αM and αE, contain a highly conserved I domain of 200 amino acid residues inserted near the amino-terminus of the subunit [2]. These I domains adopt a Rossman-fold structure and have major ligand and cation binding sites (the metal ion-dependent adhesion site (MIDAS) site) on their surfaces [3,4,5]. They are capable to directly bind ligands which play a necessary and important role in ligand binding by the intact integrins.

Both recombinant I domains from α1 and α2 integrins can bind collagens I, collagen IV, laminin and the collagen IV fragment CB3 in a divalent cation-dependent manner but their binding profiles are quantitatively different [6]. The exact role of divalent cations in ligand binding is still unclear because it is difficult to distinguish whether the cation binding produces a ligand binding conformation in the I domain or is itself required as a bridge between ligand and the I domain. Lee et al. [7] defined 'inactive' and 'active' I domain conformations, which differ in metal ion coordination. For the 'active' conformation, direct metal ion coordination by MIDAS threonine is typical, while in the 'inactive' conformation, threonine does not coordinate the metal ion directly, but through a water molecule. Nevertheless, it has also been shown that the crystal structures of the recombinant αL I domain in the presence or absence of divalent cation are very similar and that the cation has a profound effect on the charge distribution on the cation binding face of the I domain [5]. It suggests that cation, ligand and I domain form a ternary complex [6]. On the other hand, Dickeson et al. [8] have reported that ligand binding by the α2 integrin I domain results in metal ion displacement from the I domain.

In the present work, we studied the interactions of recombinant α1 and α2 integrin I domains with cations Tb^{3+}, Mn^{2+}, Mg^{2+} and Ca^{2+}. We observed that α1 and α2 I domains bind these cations with significantly different characteristics. The binding of Mg^{2+} by the α1 I domain was accompanied by significant changes of tryptophan fluorescence which could be interpreted as a conformational change. Comparison of the α1 integrin I domain structure obtained by comparative modeling with a known structure of the α2 integrin I domain shows distinct differences in the metal ion binding sites which could explain the differences in cation binding.

2. Materials and methods

2.1. Expression and purification of I domains of α1 and α2 integrins

The *Escherichia coli* cells transformed with pGEX-4T3 vector (Pharmacia LKB, Uppsala, Sweden) containing a cDNA encoding I domains of α1 and α2 integrins (a gift from Dr D. Tuckwell, University of Manchester, Manchester, UK) were selected on LB agar containing ampicillin (50 μg/ml). An overnight culture (3 ml, *E. coli* DH5α cells transformed with expression vectors) was diluted into 1 l of fresh LB_{Amp} medium and incubated to an A_{600} of 0.8 (about 5 h) at 37°C. After addition of isopropyl β-D-thiogalactoside (0.1 mM), the culture was incubated overnight at 30°C. Cells were collected by centrifugation and resuspended in TENG buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM dithiothreitol). The cells were disrupted using a French press and then, the suspension was centrifuged at $20\,000 \times g$ for 45 min. The supernatant was loaded on a glutathione-Sepharose column equilibrated with TENG buffer and rinsed with this buffer. The fusion protein was eluted by switching the buffer to 50 mM Tris-HCl, pH 7.5, containing 10 mM glutathione [9,10]. Purified fusion protein was cleaved using thrombin (Sigma) for 4 h at 25°C (10 U/mg fusion protein) and free glutathione *S*-transferase (GST) was removed using affinity chromatography on a glutathione-Sepharose column. The purity of I domains was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%, w/v) [11] stained with Coomassie blue.

2.2. Tb^{3+} fluorescence measurements

All steady-state fluorescence measurements were performed using an Perkin-Elmer LS-5B spectrofluorimeter at 20°C. The binding of Tb^{3+} cations to the fusion proteins GST-α1 or GST-α2 integrin I domain was measured using resonance energy transfer. A stock solution of $TbCl_3 \cdot 6H_2O$ (Molecular Probes, USA) was prepared at 100 mM and stored at −20°C [8]. For Tb^{3+} fluorescence measurements, 2 μM fusion protein dissolved in 50 mM Tris-HCl, pH 7.5, was titrated with increasing concentrations of $TbCl_3$. The excitation wavelength was 285 nm, Tb^{3+} fluorescence emission was measured at 545 nm. Following each $TbCl_3$ addition, the sample was mixed and

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Abbreviations: GST, glutathione *S*-transferase; MIDAS, metal ion-dependent adhesion site

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allowed to equilibrate for 5 min prior to obtaining the fluorescence intensity value. Fluorescence intensity of the free $TbCl_3$ was subtracted as background. All binding data were fitted using the equation for a rectangular hyperbola.

The ability of Mg^{2+}, Mn^{2+} and Ca^{2+} cations to quench fluorescence of Tb^{3+} bound to the α1 and α2 integrin I domains was determined by making sequential additions of these cations (100 mM stock solutions) to cuvettes containing 2 μM α1 or α2 I domain and 70 μM $TbCl_3$ [8]. Na^+ was tested as a negative control cation.

2.3. Tryptophan fluorescence measurements

All tryptophan steady-state fluorescence measurements were performed at 20°C. The excitation and emission wavelengths were 285 and 340 nm, respectively. For tryptophan fluorescence measurements, 2 μM α1 or α2 integrin I domain dissolved in 50 mM Tris-HCl, pH 7.5, was titrated with increasing concentrations of $MnCl_2$, $MgCl_2$, $CaCl_2$ or $TbCl_3$. Following each cation solution addition, the sample was mixed and allowed to equilibrate for 5 min prior to obtaining the tryptophan fluorescence intensity value. Na^+ was tested as a control cation.

For the analysis of the tryptophan quenching process in I domains, we considered the buried tryptophan residues to be totally inaccessible to KI and the others to be accessible having the same value of the Stern-Volmer constant, K_{SV}. At such conditions, the classical Stern-Volmer equation [12] is expressed by Eq. 1:

$$F_0/(F_0-F) = 1/(f_a K_{SV}[Q]) + 1/f_a, \quad (1)$$

where F_0 and F are fluorescence intensities without and with the quencher (KI), respectively, [Q] is the concentration of the quencher and f_a is the fractional maximum of accessible protein fluorescence. From Eq. 1, a plot of $F_0/(F_0-F)$ versus 1/[Q] yields a straight line with a slope $1/(f_a K_{SV})$ and intercept $1/f_a$.

2.4. Molecular modeling of the I domain of the α1 integrin structure

A model of the I domain of α1 integrin was generated by analogy to the known crystal structure of the I domain from integrin α2β1 (Protein Data Bank code 1AOX), determined at a 2.1 Å resolution by Emsley et al. [3]. The model was built and examined using the program MODELLER [13]. After the model building, a minimization procedure using CHARMM22b force field [14] was followed. All calculations were performed with the software package TINKER [15,16] running on a PC (350 MHz AMD-K6 3D, 64 MB RAM) under Linux.

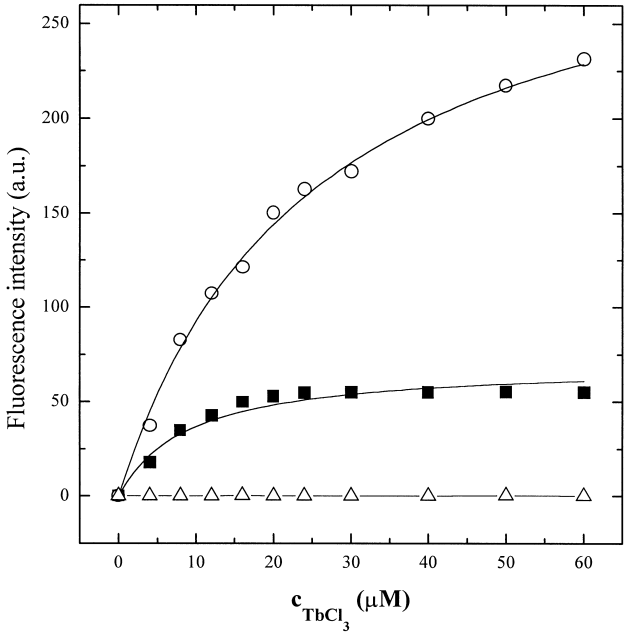

Fig. 1. Binding of Tb^{3+} to the fusion proteins GST-α1 I domain (■) and GST-α2 I domain (○). 2 μM fusion proteins dissolved in 50 mM Tris-HCl, pH 7.5, were titrated with increasing concentrations of $TbCl_3$ at 20°C. Excitation and emission wavelengths were 285 nm (tryptophan excitation maximum) and 545 nm, respectively. Free GST was tested as control (△). Fluorescence of free $TbCl_3$ was subtracted as background.

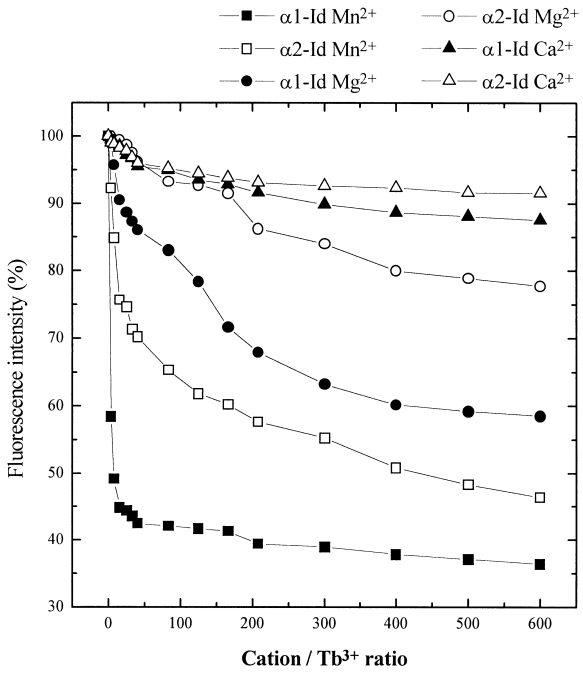

Fig. 2. Divalent cation-mediated quenching of fluorescence from Tb^{3+} bound to the fusion proteins GST-α1 I domain and GST-α2 I domain. The concentrations of fusion proteins were 2 μM and the concentration of $TbCl_3$ was 70 μM. Excitation and emission wavelengths were 285 nm (tryptophan excitation maximum) and 545 nm, respectively.

3. Results

3.1. Tb^{3+} binding to α1 and α2 integrin I domains

The Tb^{3+} cations binding to the I domains was measured using resonance energy transfer. The fusion proteins GST-α1 I domain and GST-α2 I domain were titrated with increasing concentrations of $TbCl_3$ in 50 mM Tris-HCl, pH 7.5, at 20°C and the fluorescence intensity of Tb^{3+} was determined when an equilibrium was reached (about 5 min at each step). We observed that Tb^{3+} bound to the I domains in a concentration-dependent and saturable manner (Fig. 1). The K_d of Tb^{3+} for the α1 integrin I domain was 9 ± 3 μM and for the α2 integrin I domain, it was 26 ± 4 μM. Since the I domains were expressed as GST fusion proteins, the influence of free GST on Tb^{3+} fluorescence was measured over a similar range of concentrations and no effect was observed.

3.2. Quenching of Tb^{3+} bound to α1 and α2 integrin I domains

We tested the ability of Mn^{2+}, Mg^{2+} and Ca^{2+} cations to quench Tb^{3+} fluorescence from the fusion proteins GST-α1 I domain and GST-α2 I domain (Fig. 2). We observed that both Mg^{2+} and Mn^{2+} (but not Ca^{2+}) cations were capable to quench the fluorescence of Tb^{3+} bound to α1 and α2 integrin I domains. The quenching of Tb^{3+} fluorescence from the α1 I domain was more efficient in comparison with the α2 integrin I domain. For both I domains, Mn^{2+} cations were a much more effective quencher than Mg^{2+} cations. The effect of control Na^+ cations on Tb^{3+} fluorescence was also meas-

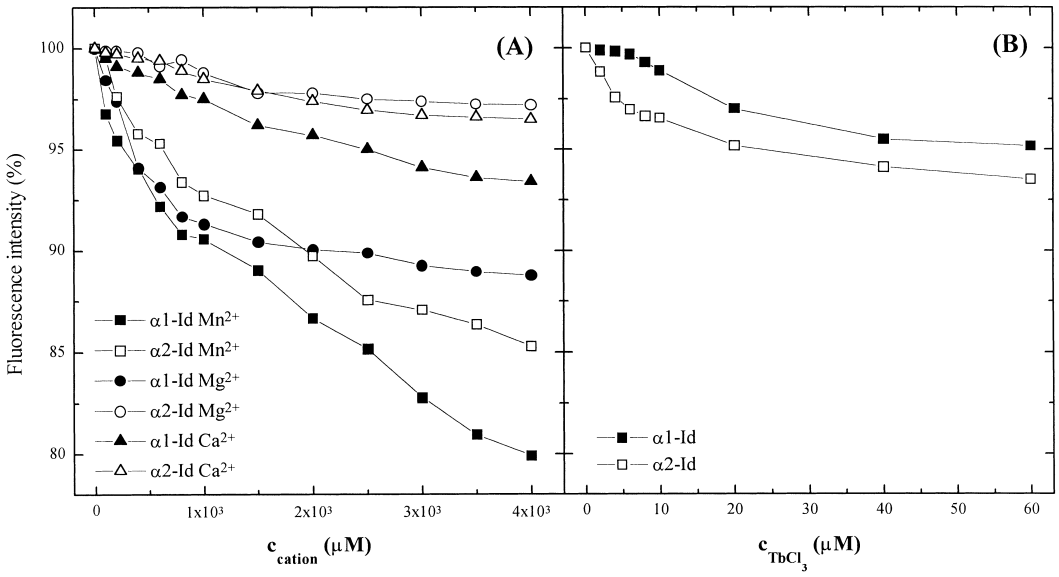

Fig. 3. Divalent cation- (A) and Tb^{3+} (B)-mediated quenching of tryptophan fluorescence of α1 and α2 integrin I domains. The concentration of I domains was 2 μM. Excitation and emission wavelengths were 285 nm (tryptophan excitation maximum) and 340 nm, respectively.

ured over a similar range of concentrations and no quenching was observed (data not shown).

3.3. Effect of cations on the tryptophan fluorescence of I domains

We also studied the effect of cations Mg^{2+}, Mn^{2+}, Ca^{2+} and Tb^{3+} on the tryptophan fluorescence of both I domains (separated from GST by thrombin cleavage) which contain only one tryptophan residue (Fig. 3A and B). We observed a significant decrease in the tryptophan fluorescence intensity for both I domains titrated with $MnCl_2$ and for the α1 I domain titrated with $MgCl_2$. The quenching of α1 I domain tryptophan fluorescence by Mg^{2+} had a saturable manner while the tryptophan quenching of both I domains by Mn^{2+} revealed no saturation. The fluorescence of Trp^{162} of the α1 I domain was always more influenced in comparison with Trp^{159} of the α2 I domain. The Tb^{3+} cations had a very weak effect on the quenching of I domains tryptophan fluorescence (Fig. 3B). In this case, the decreases in the fluorescence intensity were probably mainly due to the resonance energy transfer from tryptophan to Tb^{3+} cations.

3.4. Dynamic quenching of tryptophan fluorescence by KI

In order to test the accessibility of tryptophan residues of I domains, we performed a dynamic quenching of tryptophan fluorescence by the polar quencher KI. Stern-Volmer plots showed that Trp^{162} of the α1 I domain and Trp^{159} of the α2 I domain have significantly different quenching constants: 2.79 ± 0.05 and 1.74 ± 0.04 M^{-1}, respectively (plots not shown). Quenching data analysis also revealed that α1 and α2 I domains differ in the fractional maximum of accessible protein fluorescence: for α1 and α2 I domains were observed, $f_a = 100 \pm 2\%$ and $76 \pm 3\%$ of accessible tryptophan fluorescence, respectively.

3.5. Comparative modeling of the α1 integrin I domain structure

The sequence alignment between α1 and α2 integrin I domains is shown in Fig. 4, where the selected amino acids indicate residues involved in metal ion coordination [3]. Sequence identity is 51% and the sequences can be aligned with no gaps. It means that the α2 integrin I domain crystal structure provides a good main chain model for the α1 I domain. We created a structural model of the α1 I domain (with the sequence from Gln^{146} to Ala^{337}) using a comparative modeling approach. The final model of the α1 I domain has 96.4% of residues in the two most favored regions of the Ramachandran plot and an acceptable overall geometry, both determined with the Procheck program [17]. The overall structures of α1 and α2 I domains are similar and can be aligned with an overall root mean square deviation in 164 Cα positions of 1.14 Å.

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                146                153                163                173
α1-Id     . . .QLDIVIV       LDGSNSIYPW         DSVTAFLNDL         LKRMDIGPKQ
                  * * *        * *******         * *   **               ****
α2-Id     CPSLIDVVVV         CDESNSIYPW         DAVKNFLEKF         VQGLDIGPTK
          140                150                160                170

                183                193                203                213
α1-Id     TQVGIVQYGE         NVTHEFNLNK         YSSTEEVLVA         AKKIVQRGGR
          ****   **          *     ****         *    **  **               * **
α2-Id     TQVGLIQYAN         NPRVVFNLNT         YKTKEEMIVA         TSQTSQYGGD
          180                190                200                210

                223                233                243                253
α1-Id     QTMTALGTDT         ARKEAFTEAR         GARRGVKKVM         VIVTDGESHD
            * *              *** *   *          * **   ***         * ********
α2-Id     LTNTFGAIQY         ARKYAYSAAS         GGRRSATKVM         VVVTDGESHD
          220                230                240                250

                263                273                283                293
α1-Id     NHRLKKVIQD         CEDENIQRFS         IAILGSYNRG         NLSTEKFVEE
             ** **           *    ** **         ** **  **           * *      *
α2-Id     GSMLKAVIDQ         CNHDNILRFG         IAVLGYLNRN         ALDTKNLIKE
          260                270                280                290

                303                313                323                333
α1-Id     IKSIASEPPE         KHFFNVSDEL         ALVTTVKTLG         ERIFA
          ** *** * *           *******          **      ***         * **
α2-Id     IKAIASIPTE         RYFFNVSDEA         ALLEKAGTLG         EQIFSIEG
          300                310                320                330
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Fig. 4. The amino acid sequence alignment between α1 and α2 integrin I domains was obtained by running the MegAlign program in the Lasergene99 (DNASTAR, Madison, WI, USA) package. The selected residues are involved in metal ion coordination [3].

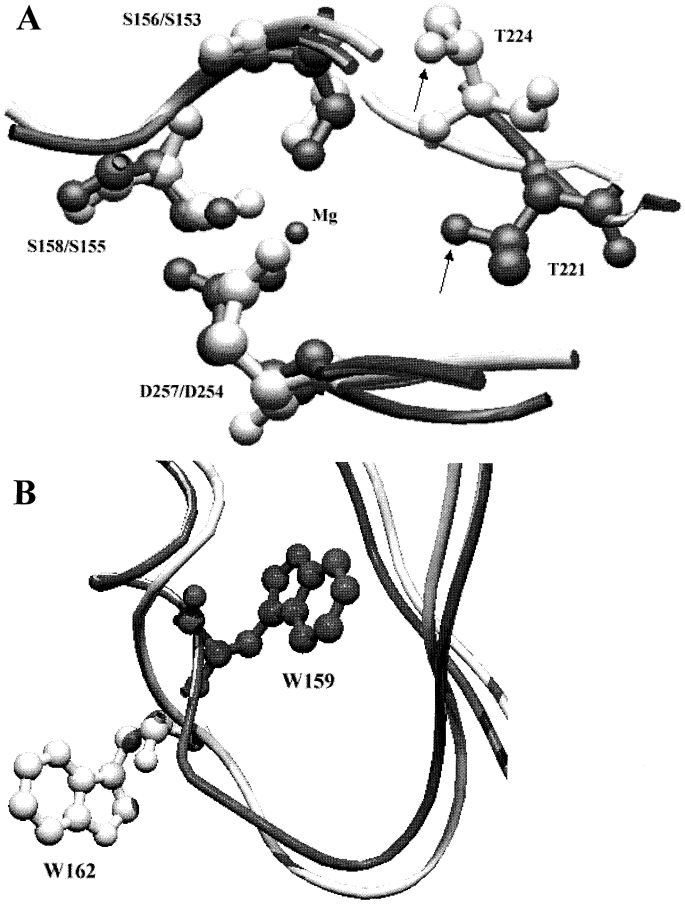

Fig. 5. (A) Comparison of metal ion binding sites of modeled α1 integrin I domain (white) and α2 I domain (dark). In the α2 integrin I domain, four amino acid residues are involved in the coordination of Mg^{2+} cation: Ser^{153}, Ser^{155}, Asp^{254} and through water molecule Thr^{221} [3]. The corresponding residues in the α1 integrin I domain are Ser^{156}, Ser^{158}, Asp^{257} and Thr^{224}. The arrows indicate distinct positions of threonine side chains.(B) Comparison of tryptophan residue positions in modeled α1 integrin I domain (white) and α2 I domain (dark).

Comparative modeling of the α1 I domain structure showed distinct alterations between the positions of Thr^{224} (α1 I domain) and Thr^{221} (α2 I domain) which are the part of the MIDAS motif. The positions of Ser^{156}, Ser^{158} and Asp^{257} (α1 I domain) are approximately similar with Ser^{153}, Ser^{155} and Asp^{254} from the α2 integrin I domain (Fig. 5A). Comparison of both structures also confirmed a different location of tryptophan residues. Trp^{162} (α1 I domain) is entirely exposed while Trp^{159} is partly buried within the protein molecule (Fig. 5B).

4. Discussion

I domains from α1 and α2 integrins follow whole integrins in the specificity and the affinity of ligand binding [6,8,19,20]. These two I domains bind a similar spectrum of ligands (collagen types I, IV, laminin) but with different affinities [6]. The ligand binding by I domains is largely divalent cation-dependent which suggests that cation, ligand and I domain form a ternary complex [6,21]. Dickeson et al. [8] proved that Tb^{3+} cations interact with a divalent cation binding site of the α2 I domain and support the binding of this domain to collagen. Specific and saturable binding of Tb^{3+} cations to the recombinant α2 integrin I domain with $K_d = 27$ μM, reported in work [8], is in good agreement with our observation ($K_d = 26$ μM, see Fig. 1). These different Tb^{3+} binding affinities for α1 and α2 I domains indicate that distinct alterations in the structure of Tb^{3+}-I domain complexes should exist.

In order to obtain more details about these differences, we tested the ability of divalent cations to quench fluorescence from Tb^{3+} bound to the I domains (Fig. 2). The observed results can be explained on the basis of Mn^{2+} and Mg^{2+} binding by I domains, because both Mn^{2+} and Mg^{2+} cations are known to support the binding of α1 and α2 I domains to collagen [6,8]. It has also been reported that Ca^{2+} cations support the binding of α1 I domain to collagen I [6]. It means that α1 I domain in contrast to α2 I domain can also bind Ca^{2+} cation which supports the hypothesis about the structural differences of these MIDAS sites. The more efficient quenching of Tb^{3+} fluorescence from α1 I domain in comparison with α2 I domain suggests that the complex α1 I domain-Tb^{3+} is more unstable than α2 I domain-Tb^{3+}. The quenching of Tb^{3+} fluorescence by Mn^{2+} and Mg^{2+} can be the result of several processes, e.g. the direct quenching of Tb^{3+} fluorescence, the displacement of Tb^{3+} from the MIDAS site and thus away from the tryptophan involved in resonance energy transfer or (mainly in the case of Mn^{2+}) the quenching of the tryptophan-excited state which serves as a source of excitation energy for Tb^{3+}. Therefore, we measured the effects of divalent cations and Tb^{3+} on tryptophan fluorescence of both I domains (Fig. 3). Mg^{2+} ions, in contrast to Mn^{2+} ions, are known as an inefficient quencher of acetyltryptophan (water

soluble tryptophan derivative) fluorescence [18]. Since the curves for titration of α1 I domain by Mg^{2+} follow a saturable manner, we suppose that these tryptophan fluorescence alterations are not the results of direct tryptophan-excited state quenching but could reflect the possible conformational changes accompanying Mg^{2+} binding to α1 I domain. Since the observed effects of Mn^{2+} on tryptophan fluorescence were certainly influenced by direct quenching of the tryptophan-excited state, it was impossible to distinguish the other effects. The different efficiencies of quenching could be caused by a different accessibility of tryptophan residues within the I domain molecules. This was proved both by dynamic quenching of tryptophan fluorescence and by molecular modeling of the α1 I domain structure (Fig. 5B).

The I domains from α1 and α2 integrins differ in their cation binding specificities and affinities as appeared from our experiments and results published elsewhere [6]. The crystal structure of the α2 integrin I domain with bound Mg^{2+} ion is already known [3]. We have used these data to build up a model of the α1 integrin I domain structure. The alignment of these two structures showed distinct differences in the MIDAS sites of α1 and α2 I domains (Fig. 5A), mainly between positions Thr^{224} (α1 I domain) and Thr^{221} (α2 I domain). The Thr^{221} of α2 I domain does not coordinate the metal ion directly, but through the water molecule [3] which is typical for an inactive conformer. It seems that this threonine is required for stability of the active conformer [18]. The different position of Thr^{224} in the α1 I domain can be the reason of its lower cation binding specificity, lower affinity to bind Tb^{3+} cations and also lower stability of the α1 I domain-Tb^{3+} complex, because the Tb^{3+} cation is in this case coordinated only by three amino acid residues (Fig. 5A) without contribution of the Thr^{224} residue.

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