NMR STUDIES OF CALCIUM-BINDING TO MUTANT α-SPECTRIN EF-HANDS

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Abstract: The co-operative calcium binding mechanism of the two C-terminal EF-hands of human αII-spectrin has been investigated by site-specific mutagenesis and multi-dimensional NMR spectroscopy. To analyse the calcium binding of each EF-hand independently, two mutant structures (E33A and D69S) of wild type α-spectrin were prepared. According to NMR analysis both E33A and D69S were properly folded. The unmutated EF-hand in these mutants remained nearly intact and active in calcium binding, whereas the mutated EF-hand lost its affinity for calcium completely. The apparent calcium binding affinity of the E33A mutant was much lower compared to the D39S mutant (~2470 µM and ~240 µM, respectively). When the chemical shift perturbations were followed upon calcium titration, a positive correlation between the D69S mutant and the binding of the first calcium ion to the wild type was revealed. These observations showed that the first EF-hand in spectrin binds the first calcium ion and thereby triggers a conformational change that allows the second calcium ion to bind to the other EF-hand.

Key Words: EF-hand, Site-Specific Mutagenesis, Multidimensional NMR

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Abbreviations used: NMR - nuclear magnetic resonance; kDa - kilodalton(s); ppm - part per million; Tris - tris(hydroxymethyl)aminomethane; HSQC - heteronuclear single-quantum correlation; NOESY - nuclear Overhauser effect spectroscopy; TOCSY - total correlation spectroscopy.
INTRODUCTION

Spectrin is a multifunctional protein with recognition sites for several other proteins. The ability to cross-link actin filaments forms the basis for the spectrin-based membrane skeleton which is associated with the cytoplasmic surface of the plasma membrane in most, if not all, cell types [1, 2]. In the red cell, the network is attached to the membrane by association of spectrin with band 3 (the anion transporter) via ankyrin and by association of protein 4.1 with glycophorin C. Several hereditary diseases have indicated the importance of a functional membrane skeleton for proper stability and shape of the red cell [3]. In other cell types it is believed that spectrin and the spectrin-based membrane skeleton restricts transmembrane proteins, such as ion channels, receptors and adhesion molecules, to requisite domains within the plasma membrane [4, 5]. The presence of sites for calcium-dependent proteins like adducin [6], calmodulin [7, 8] and calpain [9, 10] as well as for calcium ions [11, 12] together with src (SH3) [13], pleckstrin [14] and calponin homology [15] domains imply a role for spectrin in signal transduction.

Calcium and calmodulin bind to human non-erythroid spectrin ($\alpha$II$\beta$II) at sites that are either not present or degenerated in erythroid spectrin ($\alpha$I$\beta$I) [16]. Since the role of spectrin in non-erythroid cells is more diverse than in the red blood cell it is possible that calcium and calmodulin as well as other agents participate in regulatory events. For instance, a role of spectrin in mediating thrombin induced aggregation in platelets [6], secretion in adrenal chromaffin and acid secretion cells [17, 18] and transport from the endoplasmic reticulum to the Golgi [19] have been suggested. Despite the relatively low affinity, calcium binding to non-erythroid spectrin may be functional. It has, for instance, been shown that calcium and calmodulin up-regulate the proteolysis of spectrin by calpain in vitro, and its subsequent dissociation from actin [20]. More recently, it has been suggested that the binding of spectrin to NMDA [21] and $\delta$ glutamate receptors [22] is regulated by calcium. In addition, in endothelial cells spectrin, and in particular its protein 4.1 binding domain, has been shown to link calcium store depletion to calcium entry through $I_{\text{SOC}}$ [23].

The calcium-binding activity of non-erythroid spectrin has been located to the two EF-hands (EF1 and EF2) present in the C-terminus of the $\alpha$II-spectrin [24, 25]. The general calcium-binding loop of an EF-hand consists of twelve residues of which six provide ligands for the calcium ion either through their side chains, back-bone carbonyl oxygens or via a water molecule [26, 27]. The six coordinating residues are oriented around the calcium ligand in a pentagonal bipyramidal arrangement. These residues as well as several other residues both in the binding loop and in the flanking helices are well conserved in all $\alpha$II-spectrins [28-31]. Even so, the calcium binding to spectrin is much weaker (dissociation constants in the range of 100 to 500 $\mu$M [32]) than to other EF-hand-containing proteins, e.g. troponin C and calmodulin. The solution structure of an 84 amino acid peptide including the two EF-hands did not reveal any
unusual features of the EF-hand-fold of spectrin that explained the low affinity for calcium [11]. On the molecular level, calcium binding induces re-orientation of the helices flanking the binding loop, leading to the exposure of hydrophobic residues. In spectrin the structural changes are less pronounced compared to calmodulin [33] and troponin C [34] but more pronounced compared to calbindin [35]. The degree of structural changes seems to correlate with the function of calcium binding; a high degree of exposure of hydrophobic residues appears to indicate that calcium modulates interactions with other proteins [36, 37]. Based on the solution structures of the spectrin EF-hands in calcium-free and calcium-bound forms, a binding mechanism was suggested where the binding to EF1 was responsible for inducing most of the structural changes [11]. Using multi-dimensional NMR to monitor the calcium binding to a 134 amino acid peptide including the two EF-hands of spectrin, we were able to follow the chemical shifts of 25 amino acid residues during titration with calcium [12]. Factor analysis of the calcium-binding curves distinguished two distinct binding events in each titration curve, characterised by either a sigmoidal or a hyperbolic shape. Further analysis of the shapes of these two curves implied a sequential binding mechanism. The hyperbolic titration curve corresponded to the binding of the first calcium ion whereas the sigmoidal titration curve corresponded to the binding of the second calcium ion. Based on a slight predominance of sigmoidal binding curves among residues of EF1, it was suggested that the first calcium ion binds to EF2 [12]. The order of binding was also supported by experiments that showed that EF2 alone bound calcium, whereas EF1 did not [24]. However, since none of these observations provided solid evidence about the binding order of calcium ions in αII-spectrin EF-hand structures we have re-examined the binding mechanism.

To establish the binding sequence and further investigate the binding mechanism of the α-spectrin EF-hands we have used site specific mutagenesis to inactivate each of the two EF-hands. This allowed us to study the calcium binding of each EF-hand separately but still in a structural context where the structure of the unmutated EF-hand remained nearly identical to the wild type. NMR chemical shift perturbations were used to monitor structural changes in response to the mutations and to the addition of calcium [38, 39]. These measurements implied a binding mechanism where EF1 binds the first calcium ion, which triggers a structural change that allows the second calcium ion to bind to EF2. In addition, our results also revealed important sites of interactions between amino acid residues in the two EF-hands as well as a plausible explanation at the atomic level for the co-operative calcium-binding mechanism in αII-spectrin.

MATERIAL AND METHODS

Site specific mutation

Site-specific mutations were introduced into the hfSp-EF-I+II clone [24] using U.S.E Mutagenesis Kit (Amersham Pharmacia). Two mutants were prepared:
one where the glutamate at position 33 in the first EF-hand was changed to an alanine (hfSp-E33A-EF-I+II) and one where aspartate at position 69 in the second EF-hand was mutated into a serine (hfSp-D69S-EF-I+II). These mutation gave the expressed 121 residues long peptides E33A and D69S, respectively. All sequences were confirmed by DNA sequencing of both strains (Cyclist™ Exo-Pfu DNA sequencing kit, Stratagene). The sequence of the first 98 residues of the expressed wild type EF-I+II (wt-EF-I+II) is shown in Fig. 1.

R N T T G V T E E A L K

1

E n * * n n * * n X * Y* Z G -Y I -X * * -Z n * * n n * * n

EF1

D K D K S G R L N H Q E F K S C L R S L

22

33

Linker G Y D L P M V E E G E P D P

E n * * n n * * n X * Y* Z G -Y I -X * * -Z n * * n n * * n

EF2

D P N R D G H V S L Q E Y M A F M I S R

65

76

E T E N V K S S E E I E S A ...

98

Fig. 1. The amino acid sequences of the first 98 residues of the EF-I+II peptide. In the canonical sequence residues X, Y, Z, -Y, -X and -Z form the calcium co-ordination sphere. These residues, except for the residue at position -Y, which ligates calcium through its backbone carbonyl oxygen, bind the calcium ion through their oxygen bearing side chains. The residues comprising the calcium-binding loops are boxed. In the canonical sequence E is Glu, G is Gly, I is Ile, Val or Leu, n is any hydrophobic residue and * is any residue. Note that the amino acids in the two EF-hands are numbered according to how they are positioned in the EF-I+II peptide.

**Protein expression and purification**

Expression and purification of the mutants were as earlier described for the wild type EF-I+II [12]. Protein concentrations were determined from the absorbance at 280 nm using calculated molar absorptivities [40]. The purity of the expressed products was routinely checked under denaturing conditions using a Tricine-based buffer system designed for separation of low-molecular mass proteins [41]. For the mutants E33A and D69S the recovery after purification as well as the stability of the products were similar as for wt-EF-I+II.

**NMR spectroscopy and calcium titration**

For NMR spectroscopy 15N-labelled peptides were prepared by expression in M9 minimal medium [42] with 15NH4Cl as the only nitrogen source. In addition to the purification steps previously described [12], the peptides were extensively dialyzed against 0.1 M KCl, 10 mM Tris-HCl, pH 7.3 and concentrated to 1.5
mM (D69S) and 0.7 mM (E33A) using Pall Filtron or Amicon concentrators (3 or 10 kD cut-off). $D_2O$ was added to a final concentration of 5%. In order to reduce dilution of the protein samples during the calcium-titration experiments, stock solutions with different concentrations of CaCl$_2$ (2.5-1000 mM) in 10 mM Tris-HCl at pH 7.3 were used. The changes in peptide concentration during the calcium titration were accounted for in all calculations. Both one-dimensional $^1$H NMR and two-dimensional ($^1$H,$^{15}$N)-HSQC spectra were recorded at each titration point.

NMR measurements were carried out at 30°C on an AMX2-500 Bruker spectrometer operating at a proton frequency of 500.13 MHz. The $^1$H-NMR spectra were acquired using the WATERGATE pulse sequence [43]. The ($^1$H, $^{15}$N)-HSQC experiments on the $^{15}$N -labelled samples were performed by the sensitivity-enhanced gradient selection method [44]. Two-dimensional NOESY [45] and TOCSY [46] experiments were performed in the TPPI mode [47]. The water suppression in these experiments was achieved by implementation of the WATERGATE method [43].

The $^1$H and $^{15}$N resonance peaks for D69S and E33A were assigned with the help of three-dimensional NOESY-HSQC and TOCSY-HSQC experiments using an assignment strategy previously described [12]. Three-dimensional NOESY-HSQC and TOCSY-HSQC experiments were performed in the States-TPPI mode for the NOESY and TOCSY parts and in gradient enhanced selection mode for the HSQC part. Experimental and processing conditions were the same as earlier described [12].

The dissociation constants were calculated by iterative fitting of plausible binding models to the curves obtained experimentally. Since no binding could be observed to the mutated loop in neither E33A nor D69S a binding model assuming a single binding site was used for these peptides. In the iterative fitting process the fraction of the calcium-bound peptide and the free calcium concentration was calculated using the total concentrations of peptide and calcium ion and varying the dissociation constant until a best fit was obtained. All calculations were done using Microsoft Excel software package.

RESULTS

Stability and structures of D69S and E33A mutants

The expression levels, recovery after purification and stability of D69S and E33A were similar to that of wt-EF-I+II. Far-UV CD spectra of wild type and mutant peptides (not shown) were identical, indicating that the mutations did not change the $\alpha$-helical content of the peptides. The mutant peptides as well as the wild type peptide were degraded to approximately 12 kD upon prolonged storage, probably due to proteolysis of the unstructured C-terminal tail [12]. Assignments of the proton and nitrogen resonances of E33A and D69S were done by three-dimensional heteronuclear NMR experiments on $^{15}$N-labelled samples (Fig. 2). We were able to assign residues 5-85, which includes the two
Fig. 2. \((^1\text{H}, ^{15}\text{N})\)-HSQC spectra of E33A, D69S and wt-EF-I+II in the absence of calcium. Spectra were determined using 0.7 mM E33A, 1.5 mM D69S and 0.5 mM wild type EF-I+II in 10 mM Tris-Cl, pH 7.3, 100 mM KCl.
EF-hands. The amide resonances for the C-terminal amino acid residues were observed as broad signals in the central region of the HSQC spectra. These cross peaks did not have corresponding NOE peaks in the three-dimensional NOESY-HSQC spectra which further indicated the presence of multiple conformations in the incompletely folded C-terminus of each mutant. In addition, increasing amounts of degradation products were observed with time, which gave rise to sharp resonances (not shown). The spectra corresponding to the EF-hands of E33A and D69S did not change with time, indicating that only the C-terminal parts were degraded. Similar observations were made when examining wt-EF-I+II [12].

Fig. 3. Chemical shift differences between the calcium-free forms of wt-EF-I+II and D69S and between wt-EF-I+II and E33A. Chemical shifts were determined from ($^1$H, $^{15}$N)-HSQC spectra shown in Fig. 2.

The amide group proton and nitrogen chemical shift as well as the Hα chemical shift differences of wt-EF-I+II compared to E33A and D69S are shown in Fig. 3. It was apparent that the differences of the calcium-free forms were located almost exclusively to residues close to the site of mutation. This indicated that the overall structures of the mutated peptides remained intact and very similar to wt-EF-I+II. The differences observed in the rest of the molecule were generally small ($^1$HN $\Delta \delta_{ppm} <$ 0.1 ppm, $^{15}$N $\Delta \delta_{ppm} <$ 1 ppm, Hα $\Delta \delta_{ppm} <$ 0.1 ppm) and mapped to residues sequentially and structurally close to the site of mutation. For example, the E33A mutation caused amide proton chemical shift
perturbations also at the beginning of the EF1 loop (residues 22-24). This can be explained by the disruption of the salt-bridge between Lys23 and Glu33 [11]. Similarly, in D69S residues Ser15, Leu29 and Cys37 displayed significant chemical shift perturbations in the α-protons ($H_\alpha \Delta\delta_{ppm} > 0.2$ ppm) in addition to those of residues close to the site of mutation. The inter-loop interactions between Ser15 and Leu74, Leu29 and Val72, and Leu29 and Cys37 [11] are probably responsible for the observed shift perturbations caused by the D69S mutation.

**Correlations of chemical shift changes upon calcium-binding between mutant and wild-type EF-hands**

The process of calcium binding by EF-hand proteins often includes an extensive conformational change in the loop region of the EF-hand, and in some cases also a significant re-orientation of the α-helices flanking the binding loop [36]. NMR chemical shifts are sensitive to such structural changes and can therefore be used to monitor the calcium-binding mechanism. Fig. 4 shows the chemical shift perturbations upon calcium binding to the D69S and E33A mutants and wt-EF-I+II. The chemical shift perturbations of residues in the unmutated loops were similar to those observed in wt-EF-I+II. This implies that the structural changes induced by calcium binding to the mutants were similar to those observed in the corresponding loop of wt-EF-I+II. The absence of any shift perturbations of most residues in the mutated loops of D69S and E33A clearly indicated a complete loss of binding affinity of these deactivated loops. It is noteworthy that although the chemical shifts of most residues in the mutated loops of both E33A and D69S were unaffected by calcium, three residues in D69S were indeed affected by calcium. Addition of calcium caused chemical shift changes in the amide proton of Leu74 and α-protons of His71 and Ser73. The sign and size of these perturbations were very close to those observed in wt-EF-I+II.

A more systematic comparison of the structural changes was performed by correlating the present results of the mutants with previous results obtained from a factor analysis of calcium binding to wt-EF-I+II [12]. We have previously shown that the calcium-titration curves of wt-EF-I+II, as seen by chemical shift perturbations of 25 amino acids, can be described by two functions: one hyperbolic and one sigmoidal. Further analysis of the calcium-titration curves revealed a highly co-operative and thus sequential type of binding [12]. When described as a sequential order of two binding events, the hyperbolic function corresponds to the binding of the first calcium ion and the sigmoidal to the second calcium ion. In the factor analysis the relative proportions of these two binding events in each titration curve were extracted, making it possible to decompose the overall chemical shift perturbations of each residue into two components related to the first and second binding events. For example, assuming that the total chemical shift perturbation upon calcium
Fig. 4. Chemical shift changes induced by binding of calcium to E33A, D69S and wt-EF-I+II. Chemical shifts induced by addition of calcium (final calcium concentrations: wt-EF-I+II: 7.5 mM, D69S: 38 mM and E33A: 30 mM) were determined from ($^{1}H$, $^{15}N$)-HSQC spectra.
binding for the amide proton of a residue was 1 ppm and that factor analysis revealed that the proportions of the hyperbolic and sigmoidal factors were 0.8 and 0.2, respectively. Then the chemical shift changes that relate to the first binding event would be 0.8 ppm and that of the second binding event would be 0.2 ppm. It is expected that the mutant which retains an active EF-loop identical to the first binding loop in the wt-EF-I+II should have similar chemical shift changes to those related to the hyperbolical factor (first binding event) of the wt-EF-I+II.

Fig. 5. Correlations of the chemical shift changes between D69S and wt-EF-I+II (left) and between E33A and wt-EF-I+II (right). Chemical shift changes in the amide protons were obtained from Fig. 4 for the following residues: Phe14, Ser15, Met17, Phe21, Asp22, Lys23, Asp30, Phe34, Lys35, Ser36, Cys37, Asp44, Leu45, Phe57, Ala59, Val64, Arg68, Gly70, Gin75, Ala79 and Glu85.

In Fig. 5 the correlation between the chemical shift perturbations of the two mutants and those of wt-EF-I+II induced by the binding of the first calcium ion is shown. Residues in the mutants with chemical shifts that differed significantly from the wild type (such as Ser15, Leu29 and Cys37) were excluded from the analysis. D69S and wt-EF-I+II showed a very high correlation (correlation coefficient = 0.90), whereas no obvious correlation was observed for E33A and wt-EF-I+II. These data show that the calcium binding properties of the active EF-loop (EF1) of D69S is similar to the loop in wt-EF-I+II which binds calcium initially. The results therefore imply that the EF1-loop in wt-EF-I+II binds the first calcium ion, and that this binding event triggers a conformational change that leads to binding of the calcium ion to the EF2-loop.

**Calcium binding affinity of mutants and wild-type EF-hands**

Upon calcium addition it was possible to follow the chemical shifts of several amino acid residues throughout the titration procedure. The chemical shifts of all residues resolved followed a hyperbolic pattern (Fig. 6) which correlates with the fact that only one binding-loop has affinity for calcium. Therefore to analyse the titration curves of D69S and E33A we used a single-site binding model:
\[ Y = \frac{[Ca]}{K_d + [Ca]} \]

where \( Y \) is the fraction of the calcium-bound protein, \( K_d \) is the dissociation constant and \([Ca]\) is the free Ca\(^{2+}\) concentration. Best fits were obtained for dissociation constants of \(~240\ \mu M\) and \(~2470\ \mu M\) for D69S and E33A, respectively (Fig. 6), which is much weaker than the binding \(~50\ \mu M\) to wt-EF-I+II [12]. However, the calcium affinity of D69S, with an active EF1 binding loop, was less affected than that of E33A, with a deactivated EF1 binding loop. Therefore it seems likely that the initial calcium-binding event involves EF1. This corroborates the chemical shift correlation analysis that indicated this order of calcium binding in wt-EF-I+II.

**DISCUSSION**

**Mutational design and structural changes upon mutation**

The three-dimensional structure of the spectrin EF-hand domain is characterized by interactions within each binding loop as well as between the two calcium-binding loops. Due to a salt bridge between Lys22 and Glu33 the EF1-binding loop is more rigid whereas the absence of any interactions within the EF2-binding loop creates a less rigid and more open structure [11]. However, the calcium-binding loops form substantial inter-loop interactions, mainly through short anti-parallel \(\beta\)-strands, formed by Arg28-Leu29-Asn30 in EF1 and His71-Val72-Ser73 in EF2, and hydrophobic interactions between Leu29 and Val72. Leu29 and Val72 are also part of the hydrophobic core, which includes several residues from the flanking \(\alpha\)-helices.
To investigate the roles of the inter-loop and intra-loop interactions in calcium binding to human αII-spectrin critical residues in the binding loops were mutated. In EF1 the residue at position -Z (Glu33) and in EF2 the residue at position Z (Asp69) were mutated to alanine and serine, respectively. Since these residues are located away from the β-strand, these mutations should affect inter-loop interactions minimally. The importance of proper intra-loop interactions have been shown for a number of EF-hands [48-51], and it was expected that mutating one calcium-binding residue should disrupt important hydrogen bonds within the loop and thus change the loop structure. In particular, mutating the well-conserved glutamate in the -Z position has been shown to strongly affect the loop structure and in some cases also the co-operativity present within some EF-hand pairs [52].

In E33A significant changes in chemical shifts were only observed for residues within the EF1-loop. The substitution of glutamic acid for alanine affected the chemical shifts of residues close to the site of mutation as well as certain residues further away but still within this EF-hand. The observed changes in chemical shifts of some residues (i.e. Leu11, Phe14, Asp22-Lys33-Asp24, Leu29-Asn30-His31-Gln32-Glu33-Phe34, Leu41, Gly42 and Asp44) indicate, as has been suggested [11], that the salt-bridge between Glu33 and Lys23 is important for the stabilization of the loop structure in the calcium-free form. When this interaction is broken, the EF1-loop becomes less rigid, causing rearrangements of residues within the EF1 loop as well as in residues in the adjacent α-helices. The large chemical shift of the amide proton of Asn30 implied formation of a new hydrogen bond. With the exception of the residues in the β-strand, the Glu33 mutation did not cause any major chemical shift changes in the residues of the EF2-hand, strongly indicating that the mutation did not induce any structural changes in EF2.

Similarly, in D69S the major chemical shift changes were observed for residues close to the site of mutation, although some additional residues (i.e. Ser15, Leu29 and Cys37) were also affected. Considering the close contacts between the β-strand and the hydrophobic core it was not surprisingly that those changes were seen in residues in contact with these two substructures.

Binding of calcium to both mutants induced chemical shift changes in residues of the unmutated binding loop that did not differ significantly from the shift changes observed in the wild type. Therefore it seems reasonable to assume that neither mutation affected the structure of the other unmutated EF-hand to any larger extent.

**Calcium binding properties of mutant EF-hands**

From our data it is clear that the mutations did affect the calcium-binding properties of αII-spectrin; a mutated EF-hand lost its affinity completely whereas the other unmutated EF-hand remained active. The affinity of D69S, with a mutated and deactivated EF2, was nearly 10 times stronger than that of E33A, with a mutated and deactivated EF1. Based on a slight predominance of
the hyperbolic factor in residues in the EF2 loop it was previously suggested that the EF2 loop probably binds calcium first [12]. However, since the difference in binding affinities of the two mutants is a consequence of the binding order of the two EF-hands, the present results implies that EF1 binds before EF2. Further, upon calcium binding to the mutants, chemical shift perturbations for a few residues in the mutated loop of D69S but not E33A were observed. Therefore, it seems reasonable to suggest that these residues are key residues through which calcium binding of one EF-hand modulates the affinity of the other. Since residues of EF2 responded to calcium binding to EF1, and not vice-versa, the binding to EF1 triggers the binding of the second calcium ion by EF2. Moreover, when the chemical shift changes of the mutants were correlated with the hyperbolic fraction of the corresponding shift changes of the wild type (Fig. 5), there was a strong correlation between D69S and the wild type. Unlike D69S, the correlation between E33A and the wild type was much poorer. Thus multiple NMR data on the mutant peptides of α-spectrin suggest that the primary calcium binding to α-spectrin occurs in the EF1 loop.

It is also evident that the previously used approach to interpret the factor predominance to define binding order was misleading. In the system with two adjacent binding loops both loops are sensitive not only to the first binding event but also to the second binding event. Therefore when one loop binds, the residues of the other loop are also perturbed and these changes (factor propensities) are not necessarily smaller than the changes induced directly by calcium binding.

The affinities for calcium of the mutants were reduced considerably compared to wt-EF-I+II. The dissociation constants determined for the E33A and D69S mutants were ~2470 µM and ~240 µM, respectively, compared to an affinity of ~50 µM for binding of the first calcium ion to the wild type peptide. The reduced binding affinity can be due to broken co-operativity between the two EF-hands or a consequence of minor structural changes. It has been suggested [53] that the residue in the -X position, the gateway residue, is of primary importance for the binding affinity of an EF-hand motif, since it is located at the entrance of the metal binding cavity. If the gateway residue is polar, an increase in negative charge close to the entrance is expected to increase the stability of the bound metal [53]. In the calcium-free wild type α-spectrin (Fig. 7a), the side chain of the gateway residue, Asn30, and the side chains of His31, Asp69 and His71 are close enough to create a number of stabilizing interactions which would defined the overall charge of the gateway residue. In the absence of the negatively charged Asp69, the positively charged His31 and His71 decrease the overall negative charge around the gateway residue, which could lead to a reduced affinity for calcium, exactly as observed for D69S.

**Molecular basis for the binding order**

The binding of D69S represents the first stage of conformational changes that occur in α-spectrin upon binding of calcium. Since some residues in EF1 hinders
EF2 from binding calcium before EF1 is filled, any chemical shift perturbations of residues in the EF2-loop of D69S upon calcium binding would indicate the location of these constrained residues. In D69S as well as in the wild type, a substantial chemical shift (>1.1 ppm) was observed for the amide proton of Leu74 (Fig. 6) whereas the chemical shift changes of the alpha-proton and amide nitrogen of the same residue were not as distinct. However, the alpha-protons of His71 and Ser73 in EF2 exhibited noticeable perturbations, similar to those found in the wild type (Fig. 6). In E33A, the chemical shifts of amide proton of Leu74 and alpha-proton of Ser73 did not change upon calcium titration, further suggesting that Leu74 and its neighbouring residues are indeed involved in inter-loop interactions responsible for the co-operative mechanism of calcium-binding by α-spectrin EF hands. It is noteworthy that Ser73 is the gateway residue [53] of EF2 and involvement of Ser73 in inter-loop interactions, which can be modulated by the structural changes in EF1, may therefore be important for the binding properties of EF2.

To further understand the role of Leu74 and Ser73 in the inter-loop interactions and in the co-operative binding mechanism we have examined the 3D structures of calcium-free and calcium-bound forms of α-spectrin. In the calcium-free form the side chain of Arg28 covers Leu74 and Ser73 (Fig. 7a). This most probably occurs due to formation of a salt-bridge between the side chains of Arg28 and Glu76. This interaction is possible as the distance between these residues is shortened as a result of formation of an extended β-strand between the two loops. The presence of such an extended β-strand was confirmed by the NOE between the amide proton of Leu74 and the alpha-proton of Gly27 and can also be seen in the structure (Fig. 7a). A similar β-structure was also found in the N-terminal EF-hands of troponin C [54].

There is another important feature of the calcium-binding loops, which may explain why Glu76 binds Arg28. According to Strynadka and James [54] a basic residue between the X and Y positions in the canonical loop sequence usually has a stabilizing effect on the calcium-free form by formation of a salt bridge with the residue in the -Z position. In the EF1 loop of α-spectrin this stabilisation is accomplished by a salt-bridge between Lys23 and Glu33, whereas in EF2 the corresponding residue Pro66 is unable to form a salt bridge with Glu76. Therefore in absence of such a basic residue in EF2, this function is most probably carried out by the basic residue Arg28 from EF1. In contrast to the calcium-free form, Leu74 is distant from Gly27 in the calcium-bound form, the β-strand is shorter and the distance between the side chains of Arg28 and Glu76 is increased (Fig. 7b). Subsequently the side chain of Arg28 no longer restrains Leu74 and Ser73 and this segment of EF2 becomes more accessible to solvent and calcium. It is important to note that Fig. 7b represent a structure with two bound calcium ions, whereas the structural changes causing the chemical shift perturbations observed in D69S and E33A are the result of the binding of a single calcium ion.
In summary, chemical shift changes and 3D structures suggest that in the calcium free form Ser73, the gateway residue of EF2 is blocked from binding calcium by the side chain of Arg28 which is involved in a salt-bridge with Glu76. When calcium binds to EF1 these inter-loop interactions are disrupted or changed and consequently facilitates the binding of calcium to EF2.

Fig. 7. The calcium binding loops of α-spectrin in the calcium-free (top) and calcium-bound (bottom) forms. The figures are based on the structural determination of the EF-hands in chicken α-spectrin [11]. Colour code: red - negative side chains; blue - positive side chains; grey - neutral side chains; yellow - backbone.
The physiological consequence of calcium binding to α-spectrin

The affinity for calcium of the α-spectrin EF-hands are of the order of 50-100 µM, which is unusually low for calcium-binding to active EF-hand proteins. Since EF1 binds the first calcium ion the reason for this should be found within the loop architecture of this EF-hand. In EF1 of α-spectrin the 10th position is a histidine (His31) and not as usually observed in EF-hand pairs, a hydrophobic or polar/acidic residue [55]. The presence of a basic residue in this position reduces the negative charge of the gateway residue of EF1 (Asn30) and may therefore also lower the calcium affinity of EF1.

The extent of the close-open transition of EF-hands is different for different proteins, ranging from large changes (calmodulin [33] and troponin C [34]) to fairly small changes (calbindin [35]). In this context, spectrin is classified as a calcium-binding protein which undergoes moderate conformational changes [36]. Since proteins that undergo significant conformational changes upon calcium binding also seem to regulate other proteins in a calcium dependent manner, this might also be the case for spectrin. It has been shown that the interaction between NMDA and δ glutamate receptors with spectrin is regulated by calcium [21, 22]. Due to the low calcium affinity, it is expected that the calcium regulating events involving spectrin should take place in cells, or in areas of cells where the calcium concentration is at least transiently significantly increased, compared to the physiological calcium level. In addition, in respect to the conformational changes of α-spectrin EF-hands that may occur in the cell, it is possible that the calcium-bound form can be stabilized by the interaction with other proteins. This can significantly bias the measured value of binding affinity as well as the extent of conformational changes. Non-erythroid spectrin interacts not only with structural proteins, but also with proteins involved in regulatory events in the cell and it will be important to further investigate the calcium-binding properties of α-spectrin in this context.

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