

## Short communication

# Structural changes in calcium-binding allergens: use of circular dichroism to study binding characteristics

**Background:** Several studies showed that calcium-binding proteins have a fixed place in the spectrum of allergenic substances. Often the binding of a calcium ion induces conformational changes and affects immunoglobulin E-binding to the allergen. Hence, the quantitative characterization of the binding to calcium is of importance to understand both the biologic and allergenic activity of these proteins.

**Aims of the study:** In the present study we describe a procedure for determining the stoichiometry and dissociation constant ( $K_D$ ) of calcium-binding allergens using circular dichroism (CD) techniques. For the experiments, we used recombinant Bet v 4, a two EF-hand allergen from birch pollen.

**Methods:** Solutions of Bet v 4 were titrated with calcium and the change in molar ellipticity at 222 nm was monitored with a CD spectropolarimeter.

**Results:** The determination of the binding stoichiometry as well as of the  $K_D$  for one EF-hand (4  $\mu$ M) demonstrated the applicability of the method.

**Conclusions:** CD-monitored calcium-titration of protein solutions represents a fast and easy method for determining the binding characteristics of calcium-binding allergens.

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Key words: affinity; allergens; Bet v 4; calcium; circular dichroism.

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Accepted for publication 26 April 2005

A variety of calcium-binding allergens is presently known (1). Common to all of them is the presence of one or more EF-hand calcium-binding domains on their sequences. These highly conserved motifs are directly implicated in  $\text{Ca}^{2+}$ -binding of the protein. The typical EF-hand is a helix-loop-helix motif characterized by a sequence of 12 amino acids which participate in the metal ion binding (2). The first identified calcium-binding allergen was fish parvalbumin (3, 4), which contains three EF-hand calcium-binding sites, one of them being inactive. Since then, numerous allergens with different numbers of EF-hands have been described.

In many cases, it has been demonstrated that binding of calcium to the EF-hand loop induces conformational changes and affects immunoglobulin E recognition of the allergen (5–8). These results indicate that the interaction of these proteins to the physiologic ligand calcium plays an important role in both their biologic and allergenic activities. Thus the quantitative characterization of the binding to calcium represents an important part of understanding the function of this class of allergens.

In this study we show that circular dichroism (CD) can be successfully applied to determine binding characteristics of calcium binding allergens like stoichiometry and dissociation constants. Because of the low resource demands, CD measurements have been popular for

analyzing conformations or structural changes of proteins. For our experiments, we used recombinant Bet v 4 (5, 9), a 2-EF-hand allergen from birch pollen, as model allergen.

## Materials and methods

### Protein preparations

Two different protein preparations were used: the wild-type recombinant Bet v 4 (Bet v 4 WT) and a mutant Bet v 4 (Bet v 4 EF-I), in which the first EF-Hand had been disrupted. The disruptions were accomplished by single amino acid exchanges as previously described (5). The proteins were expressed in *Escherichia Coli* and purified by chromatofocusing on a PBE-94 (Amersham Biosciences, Piscataway, NJ, USA) exchanger column followed by reversed phase high performance liquid chromatography, as described in detail previously (5). The protein solutions were lyophilized and resolubilized in TBS or water and treated with Chelex 100 (Sigma-Aldrich, St Louis, MO, USA), to remove trace amounts of calcium. Protein concentration was determined by quantitative amino acid analysis using the Waters Pico-Tag system. Samples were hydrolyzed at 110°C in 6 N gaseous HCl for 14 h and derivatized with phenylisothiocyanate. Phenylthiocarbamyl amino acid derivatives were analyzed by reversed phase-HPLC (HP 1100; Agilent Technologies, Palo Alto, CA) using a  $3.9 \times 150$  mm, 4  $\mu$ m Nova-Pak C18 column (Waters, Milford, MA). UV absorbance was monitored at 254 nm.

CD-monitored  $\text{Ca}^{2+}$ -titration

Titration was monitored with a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). A 2-ml protein solution was used in cuvettes with a path length of 1 cm. For the stoichiometric analysis, titration was carried out in 20 mM TBS buffer, 150 mM NaCl, pH 7.5.  $\text{CaCl}_2$  was added in steps of 2  $\mu\text{l}$  from a 5 mM stock solution. After 1 min equilibration time, the CD at 222 nm was recorded.

For the affinity analysis, the buffer was changed to 20 mM MOPS, pH 7.5, containing 150 mM  $\text{Na}_2\text{SO}_4$  for ionic strength. As chlorine is strongly absorbing UV light, the signal becomes disturbed at low protein concentrations. Therefore,  $\text{Cl}^-$ -containing solutions should be avoided under these conditions. For that reason,  $\text{CaSO}_4$  was used for titration at low protein concentration instead of  $\text{CaCl}_2$ . On the contrary,  $\text{SO}_4^{2-}$ -containing solutions should not be used at the high concentration titration because  $\text{CaSO}_4$  precipitates are likely to form at already slightly increased protein concentrations. The calcium solution was added in steps of 2  $\mu\text{l}$  from various stocks with different concentrations. After 1 min equilibration time, the CD at 222 nm was recorded. At the low protein concentrations used (approximately 10  $\mu\text{g}/\mu\text{l}$ ), the signal was very sensitive to mixing and drifted during the titration. To compensate this effect, the protein solution was mock-titrated with distilled water previous to the measurements, and the observed signal changes were subtracted from the values obtained when titrating with calcium.

All titration experiments were repeated three to five times and the mean CD signal changes were plotted against the concentration of total  $\text{Ca}^{2+}$  in solution.

## Data analysis

Curve fitting and calculation of the coefficient of determination ( $R^2$ ) was carried out with the program Graphpad Prism (Graphpad Software, San Diego, CA, USA).

## Results and discussion

## Binding stoichiometry

Most calcium-binding allergens contain more than one EF-hand (1). However, some of these might be inactive, as observed for parvalbumin. To determine the total number of active EF-hands on the protein, an analysis of the binding stoichiometry (number of moles of calcium bound per mole of protein) has to be carried out. Previously, we showed by site-directed mutagenesis that both EF-hand motifs of Bet v 4 were able to bind calcium, and thus should theoretically bind 2 moles of calcium per mole of protein. Furthermore, calcium binding of Bet v 4 was accompanied by structural changes that could be monitored by CD. Therefore, Bet v 4 represented a good example of a calcium-binding allergen for our studies.

Titration of concentrated protein solutions results in a roughly stoichiometric binding behavior (as opposed to low concentrations, where binding equilibria closely following the law of mass action become observable), as almost every added calcium ion is bound by one of the protein molecules. As every single Bet v 4 molecule changes its conformation upon binding of calcium, the CD signal

will change during the titration until a point is reached that every molecule is saturated with calcium. We titrated a solution of recombinant wild type Bet v 4 (Bet v 4 WT) with calcium and monitored the CD signal at 222 nm ( $\Delta\text{CD}$ ), which represents the peak of  $\alpha$ -helix content. When a molar equivalent of 1.9 moles calcium per mole protein had been added, the CD signal did not change anymore (Fig. 1A). This demonstrates that Bet v 4 WT is able to bind two calcium ions. The theoretically predicted saturation of 2.0 was not obtained, probably due to minor inaccuracies in the determination of Bet v 4 WT-concentration.

The binding capacity of EF-hands can be individually tested by introducing mutations into the sequence of the remaining EF-hands of a protein. For instance, changing Asp to Ala at the first coordinating position in the EF-hand domains of Bet v 4 was shown to abrogate calcium binding. When we titrated a concentrated solution of Bet v 4 EF-I, which featured a

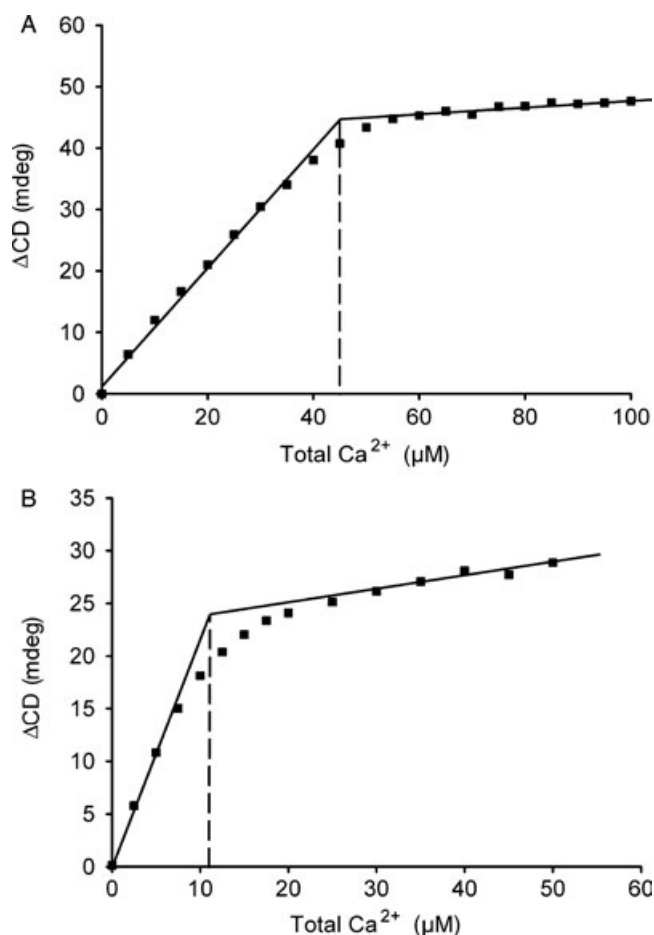


**Figure 1.** Circular dichroism monitored calcium titrations under stoichiometric binding conditions. (A) Titration of Bet v 4 WT at a protein concentration of 24  $\mu\text{M}$ . Saturation occurs at  $C_{\text{total Ca}^{2+}} = 45 \mu\text{M}$  (solid and dashed lines). (B) Titration of Bet v 4 EF-I at a protein concentration of 11  $\mu\text{M}$ . Saturation occurs at  $C_{\text{total Ca}^{2+}} = 11 \mu\text{M}$  (solid and dashed lines).

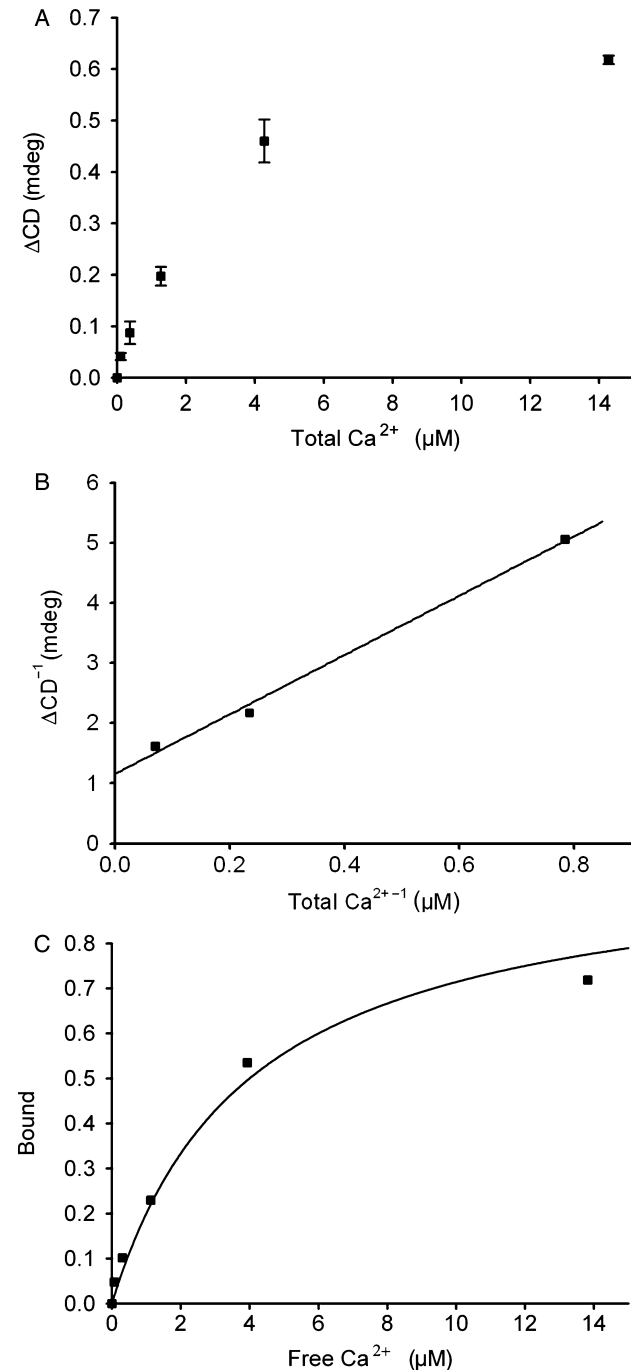


**Figure 2.** (A) Circular dichroism (CD) monitored calcium titration of Bet v 4 EF-I under equilibrium binding conditions. Protein concentration was 0.63  $\mu M$ . Error bars represent standard deviations from the mean values of three separate titrations. (B) plot of  $\Delta CD^{-1}$  vs  $C_{total} Ca^{2+}$  obtained from the titration under equilibrium binding conditions (only the nearly-linear three highest concentration data points were used). The reciprocal of the theoretically maximal signal  $CD_{Max}$  is obtained from the intersection of the extrapolated trend line (solid line) with the ordinate. This yields  $CD_{Max}^{-1} = 1.16$ . (C) saturation curve for Bet v 4 EF-I. The saturation curve obtained by curve-fitting ( $K_D = 4 \mu M$ ) is overlaid (solid line).

mutated first EF-hand, the saturation point dropped to a molar equivalent of 1.0 mole calcium per mole protein (Fig. 1B).

#### Dissociation constant

Another parameter that defines the quality of receptor–ligand interactions is the dissociation constant  $K_D$ . The  $K_D$  of an EF-hand can be obtained by carrying out titrations at low protein concentrations (a useful concentration has to be determined experimentally). This way, the equilibrium effects predicted by the law of mass action become more pronounced. Figure 2A demonstrates data points obtained by titrating a weakly concentrated solution of Bet v 4 EF-I and reading the  $\Delta CD$  at 222 nm. By plotting  $\Delta CD^{-1}$  vs  $C_{total} Ca^{2+}$  (only the nearly-linear high concentration data points were used), we obtained the reciprocal of the theoretically maximal signal  $CD_{Max}$  from the intersection of the extrapolated trend line with the ordinate (Fig. 2B).  $CD_{Max}$  was used to calculate the fractional occupancy (bound) of protein from  $\Delta CD$ , knowing from the previous experiment that Bet v 4 EF-I binds one calcium ion. Bound,  $C_{total} Ca^{2+}$  and  $C_{Bet v 4 EF-I}$  yielded the concentration of free  $Ca^{2+}$  ( $C_{free} Ca^{2+}$ ). By plotting Bound vs  $C_{total} Ca^{2+}$ , we obtained the data points for the ‘classical’ saturation curve. This saturation curve for a one-site binding model follows the formula:

**Table 1.** Calcium-binding proteins from various sources and the dissociation constant ( $K_D$ ) values for calcium of their EF-hand motifs

| Protein             | Source           | No. of EF-hand motifs | No. of calcium ions bound | EF-hand $K_D$ s                                                                                                  | Reference |
|---------------------|------------------|-----------------------|---------------------------|------------------------------------------------------------------------------------------------------------------|-----------|
| Bet v 4             | Birch pollen     | 2                     | 2                         | $K_{D1}$ (nd)<br>$K_{D2} \sim 4 \mu M$                                                                           |           |
| Calbindin $D_{28K}$ | Human brain      | 6                     | 4                         | $K_{D1} \sim 0.13 \mu M$<br>$K_{D2} \sim 0.31 \mu M$<br>$K_{D3} \sim 0.60 \mu M$<br>$K_{D4} \sim 1 \mu M$        | (10)      |
| Calgranulin C       | Pig granulocytes | 2                     | 1*/2†                     | $K_{D1} \sim 50 \mu M^*/30 nM^\dagger$<br>$K_{D2} \sim 17 \mu M^\dagger$                                         | (11)      |
| Calcineurin B       | Rat T-cells      | 4                     | 4                         | $K_{D1} \sim 24 nM$<br>$K_{D2} \sim 15 \mu M$<br>$K_{D3} \sim 15 \mu M$<br>$K_{D4} \sim 15 \mu M$                | (12)      |
| Calhepatin          | Lungfish liver   | 2                     | 2                         | $K_{D1} \sim 3 \mu M$<br>$K_{D2} \sim 170 \mu M$                                                                 | (13)      |
| Calmodulin          | Rabbit muscle    | 4                     | 4                         | $K_{D1} \sim 0.1-1 \mu M$<br>$K_{D2} \sim 0.1-1 \mu M$<br>$K_{D3} \sim 0.1-1 \mu M$<br>$K_{D4} \sim 0.1-1 \mu M$ | (14)      |
| Parvalbumin         | Carp muscle      | 3                     | 2                         | $K_{D1} \sim 0.3-1 nM$<br>$K_{D2} \sim 0.3-1 nM$                                                                 | (15)      |

\*In absence of  $Zn^{2+}$ .

†In presence of  $Zn^{2+}$ .

$$\text{Bound} = \frac{C_{\text{free Ca}^{2+}}}{K_D + C_{\text{free Ca}^{2+}}}$$

which can be easily derived from the law of mass action. The graphPad Prism Software was used to calculate a  $K_D$  to yield the best fitting curve to the calculated data points of the saturation curve (Fig. 2C). The  $K_D$  we thus obtained for the second EF-hand of Bet v 4 was 4  $\mu\text{M}$  ( $R^2 = 0.985$ ). This value lies in the medium range of binding affinities of EF-hands determined so far. Table 1 shows a comparison of EF-hand- $K_D$ s for various proteins from different sources.

The principal applicability of the method for determining stoichiometry and affinity of calcium binding is thus demonstrated. A detailed analysis of the binding characteristics of a protein requires the determination of the stoichiometry as well as the affinity of each individual EF-hand and of the whole protein. Using the method as described above, a thorough characterization can be

achieved in less than a day. In addition to speed, CD measurements have the advantage that there is no need for critical substances like fluorochromes or radioactive isotopes. The method might easily be adopted for ligands different from calcium, as the only prerequisite for a successful analysis is that the ligand itself does not give rise to a CD signal.

Concluding, we suggest that CD monitored titrations provide a convenient alternative to methods like equilibrium dialysis or fluorescence spectroscopy for the characterization of calcium binding allergens.

## Acknowledgments

This work was supported by the Joint Research Project S88-B01 (S8802-B01) of the 'Fonds zur Förderung der Wissenschaftlichen Forschung, FWF', Austria.

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