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Prokaryotic calcium-binding protein of the calmodulin superfamily

Calcium binding to a *Saccharopolyspora erythraea* 20 kDa protein

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The EF-hand calcium-binding protein from *Saccharopolyspora erythraea* has been shown, using ^{113}Cd NMR, to possess three Cd^{2+}-ion binding sites. This indicates that of the four EF-hand motifs in the molecule, one (probably site 2) is unable to bind Cd^{2+}-ions. Data from the titration of the protein with Ca^{2+}, in the presence of Quin2, were fitted to a curve calculated on the assumption that the protein contains three high affinity Ca^{2+} binding sites, two of which ($pK_1 = 8.0$, $pK_2 = 9.0$) are strongly cooperative, and one single site ($pK_3 = 7.5$). Preliminary ^1H NMR experiments indicate marked structural changes upon Ca^{2+}-binding.

Calcium binding protein; Prokaryotic; *Saccharopolyspora erythraea*; NMR; Calcium affinity

1. INTRODUCTION

The important role played by calcium ions in the regulation of a wide variety of molecular events in eukaryotic cells is well documented [1] but the role of calcium in prokaryotes has yet to be properly elucidated. Over the last decade, evidence has gradually accumulated that calcium ions are involved in diverse bacterial activities, including chemotaxis and substrate transport, sporulation, initiation of DNA replication, phospholipid synthesis and protein phosphorylation [2]. An important landmark is the recent demonstration that the intracellular Ca^{2+} concentration in *Escherichia coli* is tightly regulated to about 100 nM – a level similar to that typical of resting eukaryotic cells [3]. Furthermore, an increasing number of calcium binding proteins, some of which also have putative EF-hand Ca^{2+}-sites characteristic of the calmodulin superfamily of intracellular regulatory proteins, have been isolated in bacteria [2]. Because it occupies such a central position in Ca^{2+}-dependent regulation in eukaryotic cells, several attempts have been made to uncover a functional prokaryotic analogue in calmodulin itself. So far, the evidence for such proteins is tantalising but indirect. For example, a 21 amino acid-long stretch of the 70 kDa heat shock protein of *E. coli* [4] shows structural features typical of authentic calmodulin domains identified in eukaryotic proteins [5].

Recently, a 23 kDa calcium binding protein from *Bacillus subtilis* has been purified and characterized [6]. The protein was found to stimulate bovine brain phosphodiesterase and pea NAD kinase and reacted with anti-bovine brain calmodulin antibodies. It is not yet known if the Ca^{2+} binding sites are of the EF-hand type since the amino acid sequence has not been determined. However, the amino acid composition shows marked differences from that of eukaryotic calmodulins – for one thing, the *B. subtilis* protein is reported to possess no less than 63 Ser, 48 Gly and 11 His, while bovine calmodulin contains 4, 11 and 1 of these residues, respectively [6].

The first prokaryotic protein shown to possess a canonical EF-hand motif is a 20 kDa calcium binding protein isolated from *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*) [7,8]. It does not stimulate bovine brain phosphodiesterase, but from the amino acid sequence four potential helix–loop–helix EF-hand motifs are predicted and the overall structural organization appeared broadly similar to that observed in calmodulin. This made it tempting to ascribe a regulatory calmodulin-like function to the 20 kDa protein. It was later pointed out, however, that the *S. erythraea* protein (calerythrin) shows even more striking homology with a group of eukaryotic sarcoplasmic Ca^{2+} binding proteins [9], in which only 2–3 of the EF-hands actually bind Ca^{2+}. Intriguingly, the region of calerythrin containing the atypical EF-hand (residues 69–80) [7] bears a significant similarity to a consensus se-

Abbreviations: DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Quin2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis-(carboxymethyl)amino]quinoline; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; DFP, diisopropyl-fluorophosphate.

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quence relating members of lipocortin/calpactin family of eukaryotic Ca^{2+} binding proteins [10].

In order to characterize the 20 kDa *S. erythraea* protein and its niche in the calmodulin superfamily, we have undertaken a study of its Ca^{2+} binding properties. The calcium affinity has been measured, and the nature of the Ca^{2+} binding sites and the structural changes accompanying Ca^{2+} binding has further been characterized by ^{113}Cd and ^{1}H NMR.

2. MATERIALS AND METHODS

2.1. *Protein preparation*

E. coli K38 (pCBM1, pGP1-2) containing a plasmid over-expressing the gene for calerythrin [11], was grown as described by Swan et al. [11].

The cells were centrifuged for 6 min at 6,000 rpm and 4°C. The pellet (15 ml) was suspended in 50 ml of cold buffer A (50 mM Tris-HCl, 2 mM EDTA, 1 mM benzamidine, 1 mM DFP, 0.1 mg/ml of trypsin inhibitor, pH 7.5) and disrupted by sonication (Branson sonifier, 1/2 horn, 80% duty cycle, output control 10) in portions of 50 ml at 4°C. The mixture was centrifuged for 10 min at 20,000 rpm. The supernatant was poured off and the remaining pellet was re-suspended in 3 volumes. of buffer A, re-sonicated and centrifuged as before. The supernatant was again poured off, and the two supernatants were pooled.

The purification scheme followed that of Leadlay et al. using KCl in the place of LiCl [7]. The fractions containing calerythrin were pooled and dialyzed four times against 5 liters distilled water and lyophylized. The dried protein (130 mg) was dissolved in 20 ml of distilled water and applied to a column of Sephadex G-50 (superfine; equilibrated with buffer B) at 20°C and eluted with buffer B (50 mM ammonium acetate, pH 6.0). The fractions containing calerythrin were pooled and dialyzed against 4 × 5 liter distilled water and applied to a column of DEAE-Sephadex G-25 (equilibrated with buffer B) and lyophylized. Before and after the application of the protein solution 10 ml of 0.1 M calcium chloride solution was applied to the column to remove EDTA. Finally the protein was made calcium-free by passage through a Chelex 100 (200–400 mesh) column and lyophilized.

The purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis (gradient gel 8–25%) and IEF gel electrophoresis pH 4–6.5 using prefabricated electrophoretic plates (Pharmacia) in a Pharmacia Fast System with the standard conditions recommended by the manufacturer, and agarose gel electrophoresis [12]. Proton NMR was used to check that the protein was free of residual calcium and EDTA.

2.2. *NMR*

^{113}Cd NMR was run on a home-built 6T spectrometer at 56.55 MHz and on a Nicolet 360 WB spectrometer at 90 MHz. Both spectrometers were equipped with home-made probes with solenoidal transmitter/receiver coils for horizontal samples. The experiments were performed as described elsewhere [13]. The ^{1}H NMR spectra were run on a GE Omega 500 spectrometer at 500.13 MHz using the presaturation pulse sequence.

2.3. *Binding constants*

The Ca^{2+} binding constants were measured using the Quin2 method as described by Linse et al. [14].

3. RESULTS AND DISCUSSION

3.1. *^{113}Cd NMR*

Fig. 1 shows a ^{113}Cd NMR spectrum obtained for a sample containing approximately 1.5 mM calerythrin and 5 mM $^{113}Cd^{2+}$. As can be seen from this spectrum, there are three Cd^{2+}-ion binding sites in the protein. The Cd^{2+}-ions are bound sequentially. The two signals that appear first during the titration have chemical shifts in the range typical for normal EF-hand sites −90 to −120 ppm, whereas the third signal has a chemical shift of −135 ppm which is between that of normal EF-sites and that from the pseudo-EF-site in calbindin D_{9k} [15]. There are no additional changes in the spectrum on addition of higher concentrations of $^{113}Cd^{2+}$. An inspection of the amino acid sequence of calerythrin [7,8] shows that there are three locations with the typical 12 amino acid-long EF loop: 18–29, 113–124 and 147–158. The first of these sequences has a Gly in position 6, and position 8 is a hydrophobic residue (Leu), which is the case for all normal EF-sites; however, it does have an aspartic acid instead of a glutamic acid in position 12. This disposition of ligands is similar to that of the sarcoplasmic Ca^{2+}-binding protein from sandworm *Nereis diversicolor*. If we assume instead that this loop starts with Asp-20 there will be no Gly in position 6 and no hydrophobic residue in position 8, which is much less attractive although there will be a glutamic acid in position 12.

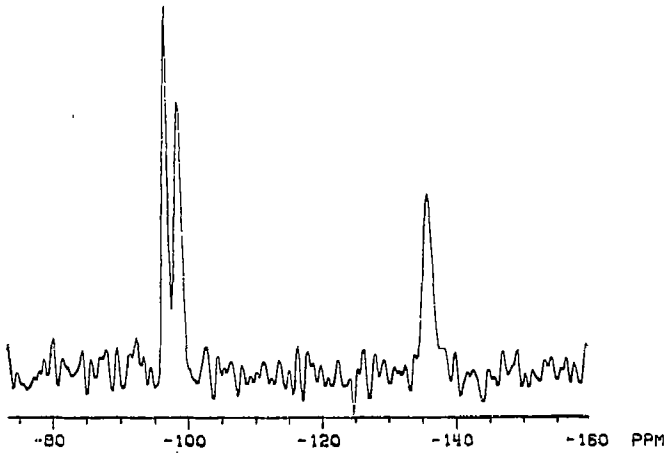

Fig. 1. ^{113}Cd NMR spectrum from a solution containing 1.5 mM calerythrin and ca. 5 mM Cd^{2+}. pH 6.0 and 27°C. Standard: 0.1 M $Cd(ClO_4)_2$.

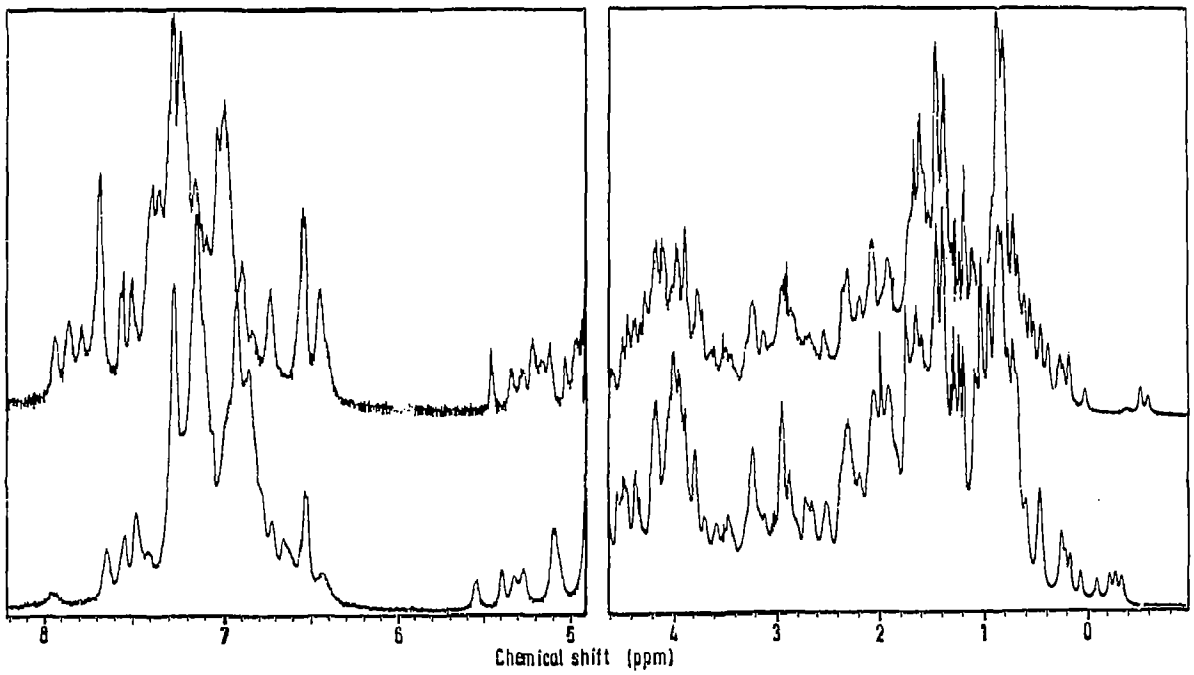

Fig. 2. Proton NMR spectra (500 MHz, 27°C, pH 6.0). Standard: 3-(trimethylsilyl)-propionic acid. Upper spectrum: 2.0 mM apo protein. Lower spectrum: 2.0 mM protein and ca. 7 mM Ca^{2+}.

Presently the ^{113}Cd resonances cannot be assigned with complete certainty to specific sites, even though we are tempted to believe that the two resonances with normal EF hand chemical shifts are due to $^{113}Cd^{2+}$-ions bound to sites 3 and 4 that may form a pair of sites, which is the normal functional unit. This leaves the third resonance for site 1, which has no 'partner' metal binding site and does deviate slightly from the typical EF-site.

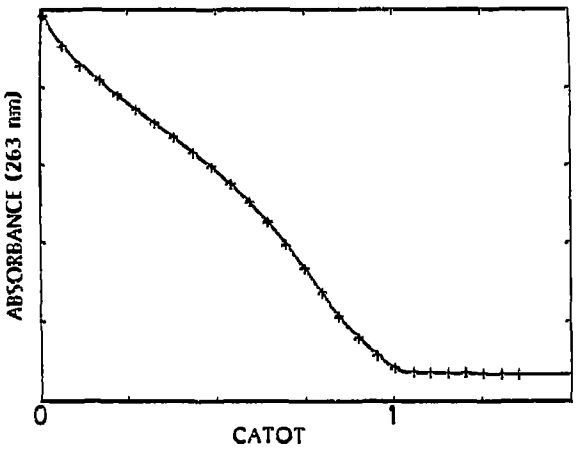

Fig. 3. Example of experimental data (+), absorbance at 263 nm vs. normalized total Ca^{2+} concentrations (CATOT) from a Ca^{2+} titration of Quin2 in the presence of calerythrin. The data have been normalized such that CATOT = 1 corresponds to total Quin2 concentration plus 3 times the protein concentration. The fully drawn curve has been calculated using the following Ca^{2+} binding constants: $\log K_1 = 8.08$, $\log K_2 = 9.10$, $\log K_3 = 7.57$.

3.2. *^1H NMR*

The proton NMR spectra shown in Fig. 2 show that there is a pronounced effect on the spectra caused by Ca^{2+} binding. It is also evident that there is an unusually large dispersion of the resonances, for example in the methyl region, with several well resolved resonances. This is most likely due to the presence of three tryptophans (Trp-18, Trp-109, Trp-128) which are more effective in causing ring current shifts than either phenylalanine or tyrosine. This also makes calerythrin, despite its size, a good candidate for 2D NMR studies and 3D structure determination and this work is in progress. Preliminary ^1H NMR studies have resulted in the assignment of three Gly residues with high field shifted NH protons, typical for Gly-6 in EF-hand calcium binding loops indicating that the first Ca^{2+} binding site indeed comprises amino acids 18–29.

3.3. *Ca^{2+} binding constants*

The Ca^{2+}-ion binding constants for calcium binding to calerythrin have been determined under low ionic strength conditions through a Ca^{2+}-ion titration in the presence of Quin2. The Ca^{2+}-binding to Quin2 was monitored by the absorbance at 263 nm as described previously [15]. The analysis of the data under the assumption of three binding sites, resulted in a pair of strongly cooperative high affinity sites ($pK_1 = 8.0$, $pK_2 = 9.0$) and one single site ($pK_3 = 7.5$). The agreement between experimental and calculated binding curves is shown in Fig. 3. There is thus one pair of strongly cooperative sites, but also strong binding to an isolated site. To our knowledge this is the first case so far observed where the

metal binding to a single EF-site is sufficiently strong to result in slow metal exchange on the NMR time scale.

4. CONCLUSION

The data presented here clearly show that calerythrin contains three high-affinity metal binding sites, two of which are most likely very similar to the archetypical EF-hands in calmodulin and troponin C. At least two of the sites show positive cooperativity. The third site appears to have a ligand disposition similar to that in the first Ca^{2+} binding site of the sarcoplasmic Ca^{2+} binding protein from the sandworm *N. diversicolor* [16]. Ca^{2+} binding is accompanied by marked changes in the ^1H NMR spectra indicating structural rearrangements. Taken together, the findings in this paper reinforce the close similarity between calerythrin and the invertebrate sarcoplasmic Ca^{2+} binding proteins [9,16]. They also provide fresh impetus to the search for a biological role for the *S. erythraea* calcium binding protein [8,11].

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