BMC Biochemistry

Research article

Ca^{2+} binding to complement-type repeat domains 5 and 6 from the low-density lipoprotein receptor-related protein

Olav M Andersen*[1], Henrik Vorum[2], Bent Honoré[2] and Hans C Thøgersen[1]

Address: [1]Laboratory of Gene Expression, Department of Molecular and Structural Biology, University of Aarhus, DK-8000 Aarhus C, Denmark and [2]Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Email: Olav M Andersen* - o.andersen@mdc-berlin.de; Henrik Vorum - hv@biokemi.au.dk; Bent Honoré - bh@biokemi.au.dk; Hans C Thøgersen - hct@biobase.dk

* Corresponding author

Published: 18 August 2003

BMC Biochemistry 2003, **4**:7

Received: 27 April 2003
Accepted: 18 August 2003

This article is available from: http://www.biomedcentral.com/1471-2091/4/7

Abstract

Background: The binding of ligands to clusters of complement-type repeat (CR)-domains in proteins of the low-density lipoprotein receptor (LDLR) family is dependent on Ca^{2+} ions. One reason for this cation requirement was identified from the crystal structure data for a CR-domain from the prototypic LDLR, which showed the burial of a Ca^{2+} ion as a necessity for correct folding and stabilization of this protein module. Additional Ca^{2+} binding data to other CR-domains from both LDLR and the LDLR-related protein (LRP) have suggested the presence of a conserved Ca^{2+} cage within CR-domains from this family of receptors that function in endocytosis and signalling.

Results: We have previously described the binding of several ligands to a fragment comprising the fifth and the sixth CR-domain (CR56) from LRP, as well as qualitatively described the binding of Ca^{2+} ions to this CR-domain pair. In the present study we have applied the rate dialysis method to measure the affinity for Ca^{2+}, and show that CR56 binds 2 Ca^{2+} ions with an average affinity of K_D = 10.6 microM, and there is no indication of additional Ca^{2+} binding sites within this receptor fragment.

Conclusions: Both CR-domains of CR56 bind a single Ca^{2+} ion with an affinity of 10.6 microM within the range of affinities demonstrated for several other CR-domains.

Background

The ligand-binding domains of the entire LDLR family of cell surface receptors are comprised of imperfect repeats of about 40 amino acids, the CR-domains [1–3]. Each repeat contains six cysteine residues which are disulphide linked in the pattern one to three, two to five, and four to six, and each repeat harbors a Ca^{2+} binding site [4,5]. Furthermore, the modular domain architecture of LRP comprises several epidermal growth factor (EGF)-precursor homology domains, a segment spanning the plasma membrane and a cytoplasmic domain. The entire LRP molecule might contain as many as 39 Ca^{2+} binding sites, one located in each of the 31 CR-domains [5–7], and two binding sites present in each of 4 EGF-domain pairs [7–11] (see legend to Figure 1A).

The understanding of how LRP binds Ca^{2+} ions is important. The binding of all ligands is dependent on the presence of Ca^{2+} [12–14] and ligand dissociation within the endocytic pathway has been suggested to occur as a possible consequence of the decrease in pH and the accompanying loss of affinity for Ca^{2+} in the acidic endosomes [8].

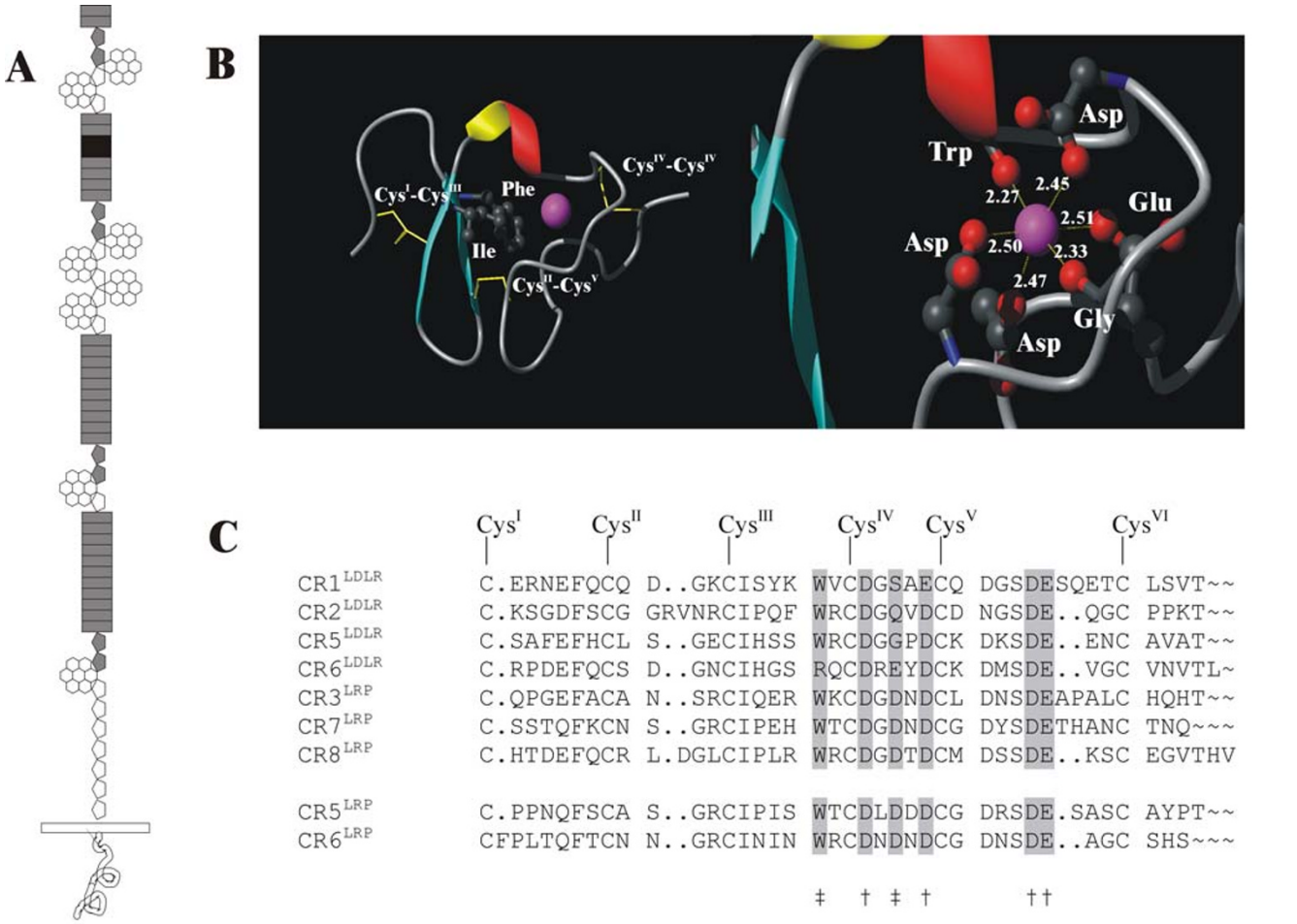

Figure 1
(A) Schematic representation of the domain architecture of the LRP, with domains suggested to contain a Ca^{2+} binding site as shown in gray. For estimation of the stoichiometric binding of Ca^{2+} to the entire LRP molecule, each CR-domain is a potential cation carrier based on the fact that all CR-domain structures described so far include a bound Ca^{2+} ion [5–7]. The crystal structure of the LDLR YWTD-repeated β-propeller does not show any cations and we assume similar lack of Ca^{2+} binding to these fragments within LRP. In contrast, some EGF-domains do bind Ca^{2+} and some do not. For LDLR the EGF-domain pair amino-terminal to the β-propeller binds 2 Ca^{2+} ions, and accordingly, it is assumed that the four EGF-domain pairs within LRP are also Ca^{2+} binding. The carboxy-terminal EGF-domain is not chelating a Ca^{2+} ion [7–11]. By these assumptions, one LRP molecules could bind a total of at least 39 Ca^{2+} ions. Rectangles (▭) represent CR-domains, pentagons (⬠) represent EGF-domains, and hexagonals (⬡) for each blade of the β-propellers. (B) Ribbon representation of the canonical CR-domain folding and Ca^{2+} binding site. *Left*, the backbone folding of CR5^{LDLR} (Protein Data Bank accession code 1ajj [5]) showing the location of the Ca^{2+} ion indicated as the sphere. *Right*, zoom of the Ca^{2+} cage showing the octahedral cation coordination. (C) Alignment of the primary structures of Ca^{2+}-binding CR-domains with a known 3D-structure, the first, the second, the fifth, and the sixth CR-domain from LDLR, and the third, the seventh, and the eighth CR-domain from LRP. The symbols below the alignment indicate residues involved in Ca^{2+} chelation (‡, coordination by backbone carbonyl; †, coordination by side chain carboxyl), and the six conserved cysteines are indicated above the sequences with roman numbers.

Ligand recognition requires key residues in the CR-domains of the LDLR-like proteins as well as the presence of Ca^{2+} ions. One important residue for this interaction is located at the center position between the fourth and the fifth cysteine residue, where an acidic side chain is important for the efficient recognition of multiple protein ligands [15,16]. The backbone carbonyl group of residues located at identical positions in several domains homologous to CR5 and CR6 is involved in the coordination of a Ca^{2+} ion [5,6]. The coordination sphere of the bound Ca^{2+}

ion is well defined in octahedral geometry, with four carboxylate oxygen atoms from the acidic motif in one plan and two carbonyl oxygen atoms completing the coordination sphere at the apices (Figure 1B).

We have previously demonstrated the specific binding of LRP ligands to a minimum receptor fragment comprising only 2 CR-domains [15], and focused on the ligand interaction with the tandem domain CR56 fragment [17,18]. The affinity for a bound Ca^{2+} ion has been reported for several CR-domains, and in order to better understand the Ca^{2+}-dependent ligand binding properties of CR56 we have determined the stoichiometry and affinity for Ca^{2+} binding to this CR-domain pair. Furthermore, we undertook a stringent/direct method of affinity determination using the microchamber rate dialysis method [19] independent of the local molecular environment at the Ca^{2+} localization site, and could compare the determined affinity with data obtained by less direct methods, e.g. fluorescence analysis. We conclude that in general the affinities do not vary significantly among the CR-domains investigated.

Results and Discussion

The demonstration of Ca^{2+} binding to the ubiquitin-fused CR56 protein [15] strongly suggested that CR56 contains at least one efficient Ca^{2+} binding site similar to other CR-domains [1,5]. However, from the previously adopted approach we could not determine the stoichiometry and affinity between Ca^{2+} and CR56. This was the main objective of the current study. Furthermore, we wanted to test whether the coupling of a CR-domain to a neighboring domain would influence the affinity of the single CR-domain, compared to the increasing pool of data describing the binding of Ca^{2+} to isolated CR-domains [6,20–23]. After affinity purification of ubiquitin-fused CR56 containing the authentic amino acid sequence, we liberated the CR56 domain pair from its fusion partner by factor X_a cleavage (as described in ref. [15]). 2D gel analysis showed a high degree of purity, since the recombinant CR56 protein migrated as a single major spot (not shown). Ca^{2+} binding was measured using the microchamber rate dialysis technique [19,24].

The Ca^{2+} binding data is shown in Figure 2A, and from Scatchard analysis of the data (Figure 2B), we conclude that the folded CR56 domain pair binds two Ca^{2+} ions with an average affinity of K_D = 10.6 μM assuming two identical binding sites. Even though there is a small deviation from the exact 1:2 stoichiometry (n = 2.0 sites), the observed n = 2.2 is within experimental error not significantly different from 2.0. Secondly, the number is in accord with other data determining Ca^{2+} affinity to tandem CR-domain pair [25]. Bieri et al. found a similar stoichiometry (n = 2.2) when they measured the Ca^{2+} binding to a concatemer of $CR1^{LDLR}$-$CR2^{LDLR}$, and neither solution nor crystal structure indicate that there is more than two Ca^{2+} binding sites within this fragment [7,26]. Furthermore, the recently solved crystal structure of the LDLR luminal domain shows a single bound cation for each CR-domain without any additional interdomain binding sites [7]. The determined affinity is very close to the reported Ca^{2+} affinities for homologous LRP CR-domains, $CR3^{LRP}$, $CR7^{LRP}$, $CR8^{LRP}$ and for the two LDLR CR-domains $CR1^{LDLR}$ and $CR2^{LDLR}$ (listed in Table 1), which indicates that the binding sites might be similar. Apparently, most CR-domains bind Ca^{2+} with an approximate affinity K_D~10–20 μM, except $CR5^{LDLR}$ and $CR6^{LDLR}$ which show a higher affinity. Furthermore, our data suggests that the various methods used to measure the affinities are reliable.

The Ca^{2+} ions in the hitherto solved domain structures are located in identical Ca^{2+} cages as for $CR5^{LDLR}$ described previously by Fass and colleagues [5] (Figure 1B). From a high level of sequence conservation for the sequences of $CR5^{LRP}$ and $CR6^{LRP}$ compared to the sequences of the CR-domains with a solved domain structure (Figure 1C), we suggest that the binding site for Ca^{2+} within both CR5 and CR6 are very similar to these. This is very important information for the assignment of nuclear magnetic resonances for the solution structure determination of CR56 (ongoing project).

The demonstration of independent folding of each CR-domain in tandem CR-domain pairs is substantiated by the reports of a total lack of interdomain interactions, and that Ca^{2+} coordination does not involve chelates from adjacent CR-domains [27,28]. In line with this our data suggest that two and only two Ca^{2+} are bound per tandem fragment as also reported for domain pairs from LDLR comprising $CR1^{LDLR}$-$CR2^{LDLR}$ and $CR5^{LDLR}$-$CR6^{LDLR}$ [25,27]. Since the Ca^{2+} affinity for CR5 and CR6 is similar to other known binding sites, it is tempting to believe that the Ca^{2+} ion is located in a similar Ca^{2+}cage as for these domains [1,5,6], and therefore the chelating residues located at identical positions within the primary structure is also cation coordinating in these domains. If this indeed is the case, we have recently demonstrated that the two residues speculated to provide electrons for Ca^{2+}coordination via their backbone functional group (Trp-953/Asp-958 in CR5 and Trp-994/Asp-999 in CR6) both contribute significantly to ligand binding [17,18]. The possibility that Ca^{2+} most likely are intimately linked to these residues suggest that Ca^{2+} binding exerts influence on ligand binding to CR-domains, because of a lack of dynamic and flexibility of residues at this particular position. In addition, especially the acidic residues is also involved in the intramolecular binding of CR-domains to the EGF-precursor homology domain at low pH, speculated to

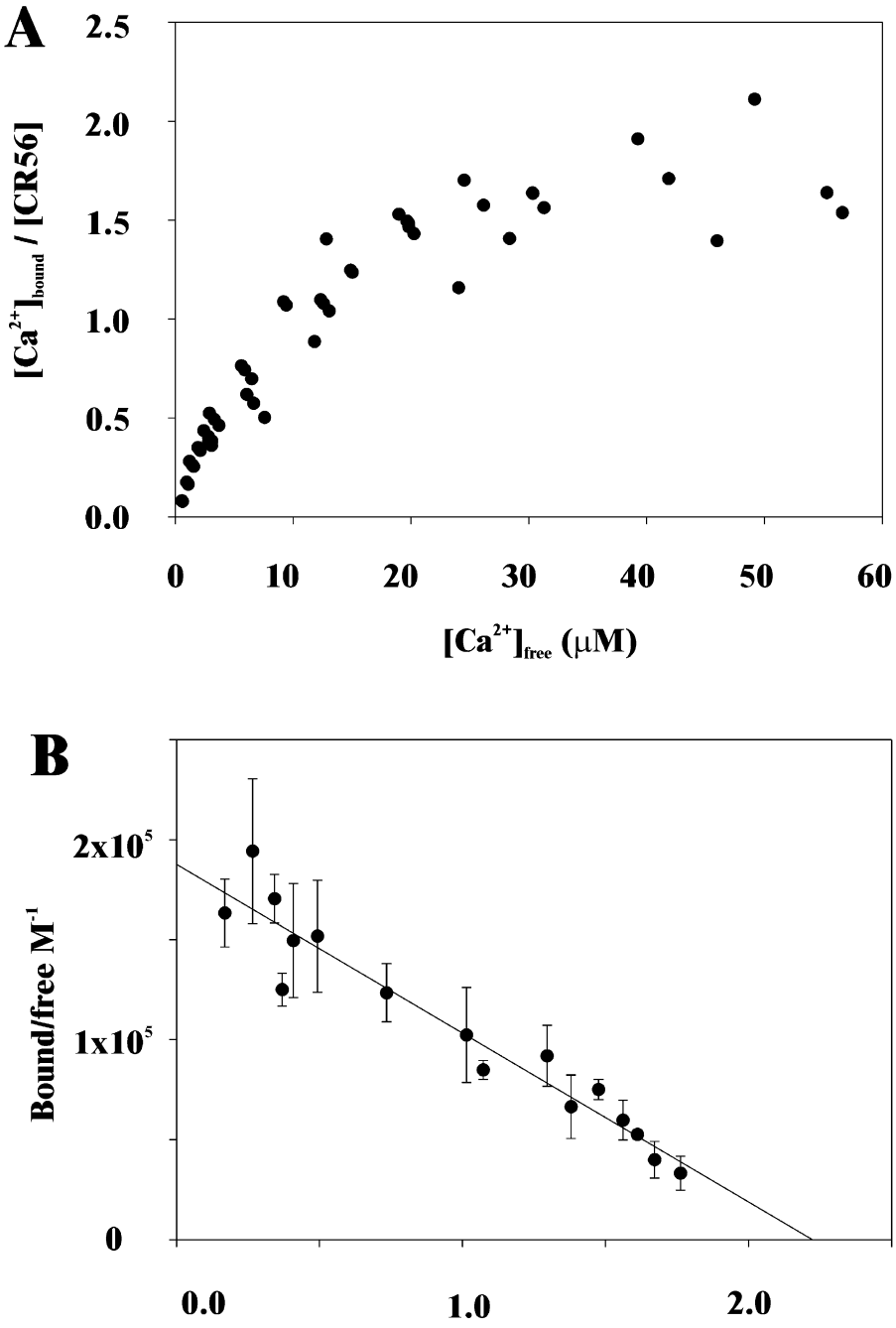

Figure 2
(A) The Ca^{2+}-affinity of CR56 was determined by rate dialysis $[Ca^{2+}]_{bound}$/[CR56] versus the free Ca^{2+} concentration. The concentration of CR56 was 15 μM throughout the analysis. (B) Scatchard analysis of the data shown in panel A. The solid line represents the best fit by least-squares analysis as described in the materials and methods section. The fact that the experimental points are located on a straight line indicates absence of cooperativity between the two Ca^{2+} binding sites. Strong positive cooperativity would result in upward curvature, while strong negative cooperativity would result in downward curvature of the Scatchard plot. The average affinity is determined to, K_D = 10.6 μM assuming two identical sites (n = 2.2).

Table 1: Ca^{2+} binding properties of various CR-domains

CR-domain	*Ca^{2+} affinity (K_D) μM*	*References*	*Method [a)]*	*Conditions*
$CR1^{LDLR}$	7	[22]	Fluorescence	25°C
	10	[30]		
$CR2^{LDLR}$	14	[25]		
$CR5^{LDLR}$	0.040	[27]	Fluorescence	pH 7.0
	0.070	[20,27]	Fluorescence	pH 7.0
	0.500	[6]	ITC	pH 7.4, 30°C
	13	[6]	ITC	pH 5.0, 30°C
$CR6^{LDLR}$	0.200	[27]	Fluorescence	pH 7.0
$CR3^{LRP}$	8	[6]	ITC	pH 7.4, 30°C
	24	[22]	Fluorescence	25°C
$CR5^{LRP}$	11	The present data	Rate dialysis	pH 7.4, 37°C
$CR6^{LRP}$	11	The present data	Rate dialysis	pH 7.4, 37°C
$CR7^{LRP}$	13	[6]	ITC	pH 7.4, 30°C
$CR8^{LRP}$	6	[6]	ITC	pH 7.4, 30°C
	13	[22]	Fluorescence	25°C
CR^{Tva}	40	[23]	ITC	pH 7.4, 30°C

[a)] ITC; Isothermal titration calorimetry CRX^{LDLR}, CRX^{LRP}, and CR^{Tva} denote the *X*th CR-domain counting from the amino-terminal end of LDLR, LRP and the Tva receptor of avian leucosis and sarcoma virus type A, respectively.

result in structural rearrangement and ligand release within endosomes, underscoring the importance of understanding the local environment around the Ca^{2+} binding site [7].

Conclusions

Both CR-domains of the CR56-domain pair bind a single Ca^{2+} ion with an average affinity, K_D~10.6 μM.

Methods

Proteins

Production and RAP-affinity purification of the ubiquitin-fused CR-domain pair comprising the fifth and the sixth CR-domain from LRP (see Figure 1) was described previously [15]. Purity was verified by two-dimensional gel electrophoresis showing the sample to be highly homogeneous (not shown).

Calcium binding analysis

Qualitative ^{45}Ca blotting analysis to ubiquitin-fused CR56 has been described [15]. The quantitative rate dialysis method [19] was applied to determine the Ca^{2+} binding constants for CR56. The binding experiments were performed in a medium containing 10 mM HEPES pH. 7.0, 150 mM NaCl and a final CR56 concentration at 15 μM. Buffers and protein solutions were passed through a Chelex 100 column (BioRAD) in order to obtain cation free solutions before use. The resin was pre-equilibrated with HEPES binding buffer before use. We have previously shown that this procedure is able to bring the Ca^{2+} content of the solutions to negligible levels as determined by atomic absorption spectrometry [29]. The dialysis membrane was of cellulose, Type 14.10, molecular cut-off 5000, from Diachema (Munich, Germany). The following equation was used to calculate the free Ca^{2+} concentration from the total Ca^{2+} concentration:

$$[Ca^{2+}]_{free} = - [Ca^{2+}]_{total} [k^*(t+t_0)]^{-1} \ln [(A_{left} - A_{right})/(A_{left} + A_{right})]$$

where k is a pre-determined rate constant, t is time of dialysis and t_0 is an experimentally determined value which is dependent upon the procedure of filling, withdrawal, and rinsing of chambers and varies with the substance dialysed [19]. A_{left} and A_{right} denote the radioactivity in the left and right solution, respectively, after dialysis measured by liquid scintillation counting in an LKB Wallac 1218 Rackbeta scintillation counter. The values of k and t_0 for the Ca^{2+} ligand used were 0.04650 min^{-1} and 0.18 min. They were determined in separate experiments with no protein present and using varying dialysis times as described in detail [19]. In short, values of ln $[(A_{left} - A_{right})/(A_{left} + A_{right})]$ are plotted versus the dialysis time. The rate constant k is then determined from the slope of the curve while t_0 is determined as the numerical value of the intercept with the time-axis (x-axis). The average number of Ca^{2+} ions bound per protein molecule, r, was calculated from

$$r = ([Ca^{2+}]_{total} - [Ca^{2+}]_{free})/ [CR56]$$

Under the presumption that CR56 contains a number, n, of identical and independent Ca^{2+}-binding sites the bind-

ing isotherm was fitted to the Scatchard equation by linear regression:

$r/[Ca^{2+}]_{free} = -r/K_D + n/K_D$,

where K_D is the dissociation constant.

Abbreviations

CR-domain, complement-type repeat domain; EGF, epidermal growth factor; LDLR, low-density lipoprotein receptor; LRP, LDLR-related protein

Authors' contributions

OMA produced and purified recombinant protein, prepared figures and drafted the manuscript. HV and BH performed the quantitative rate dialysis measurements and participated in drafting the manuscript. HCT conceived the study as well as participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

We thank Kirsten Peterslund for excellent technically assistance. This work was supported by Grant 9901730 from the Danish National Science Research Council (HCT), the Danish Medical Research Council and the Novo Nordisk Foundation.

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