

Structural Changes in the Lectin Domain of CD23, the Low-Affinity IgE Receptor, upon Calcium Binding

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Summary

CD23, the low-affinity receptor for IgE (FcεRII), regulates IgE synthesis and also mediates IgE-dependent antigen transport and processing. CD23 is a unique Fc receptor belonging to the C-type lectin-like domain superfamily and binds IgE in an unusual, non-lectin-like manner, requiring calcium but not carbohydrate. We have solved the high-resolution crystal structures of the human CD23 lectin domain in the presence and absence of Ca²⁺. The crystal structures differ significantly from a previously determined NMR structure and show that calcium binding occurs at the principal binding site, but not at an auxiliary site that appears to be absent in human CD23. Conformational differences between the apo and Ca²⁺ bound structures suggest how IgE-Fc binding can be both calcium-dependent and carbohydrate-independent.

Introduction

CD23 is a type II transmembrane glycoprotein and C-type lectin expressed in various hematopoietic cells such as mature B cells, follicular dendritic cells, activated macrophages, monocytes, eosinophils, and platelets. Several ligands have been identified for CD23, including IgE, CD21 (Aubry et al., 1992), the β2-integrins CD11b/CD18 (CR3) and CD11c/CD18 (CR4) (Lecoanet-Henchoz et al., 1997), HLA-DR (Karagiannis et al., 2001), and the α_v chain of the vitronectin receptor (Hermann et al., 1999; Kijimoto-Ochiai, 2002). CD23 binds to HLA-DR expressed in B cells, potentially enhancing antigen presentation to T cells and stabilizing subsequent HLA-TCR interactions by interacting with CD21 (Karagiannis et al., 2001). Soluble fragments of CD23 containing the lectin domain also exhibit proinflammatory activities in vitro, stimulating cytokine production through interactions with non-IgE ligands (Armant et al., 1995; Hermann et al., 1999; Lecoanet-Henchoz et al., 1997).

CD23 functions as one of two receptors for IgE antibodies. IgE binds to a high-affinity IgE-Fc receptor (FcεRI) expressed on mast cells and basophils. The aggregation of IgE:FcεRI by antigen stimulates immune responses important for defense against bacterial, viral, and parasitic infections, but it can also trigger an allergic response (Nadler et al., 2000). Allergic reactions affect up to 20% of the population in developed countries (Cookson, 1999), and the incidence of allergies and asthma

has steadily risen over the course of this century, causing significant levels of morbidity and increasing health-care costs. IgE binding to CD23 (Sutton and Gould, 1993) provides feedback regulation of serum IgE levels (Lewis et al., 2004; Payet-Jamroz et al., 2001; Yu et al., 1994) and can deliver IgE bound antigens for antigen processing or the transport of complexes across epithelial layers (Montagnac et al., 2005). Two isoforms of human CD23 (a and b) are generated through the use of different promoters and by differential splicing of exon 2 (Kijimoto-Ochiai, 2002; Yokota et al., 1992). The resulting proteins differ in 6–7 residues in the short N-terminal cytoplasmic domain, and their activities and expression are cell-type specific (Ewart et al., 2002; Montagnac et al., 2005; Yokota et al., 1992).

While FcεRI belongs to a family of immunoglobulin-like Fc receptors with specificity for distinct antibody classes (Nadler et al., 2000), CD23 is unique in that it belongs to the C-type lectin-like domain (CTLD) family of protein folds. No other antibody class has a receptor homologous to CD23. CD23 is a type II transmembrane glycoprotein and a C-type lectin. The N-terminal cytoplasmic domain is followed by the transmembrane region and then by a stalk region predicted to form an α-helical coiled coil (Beavil et al., 1992; Chen et al., 2002; Dierks et al., 1993). The C-type lectin domain sits atop the stalk. Beyond the lectin domain there is a stretch of ~30 residues whose function is not known (Figure 1).

The CTLD family is characterized by the canonical C-type lectin fold first described for the mannose binding protein (MBP) structure (Weis, 1997; Weis et al., 1991, 1998). Some CTLD-containing proteins do not bind calcium or carbohydrate but interact with other ligands, usually proteins (Kijimoto-Ochiai, 2002; Weis, 1997; Weis et al., 1998). Ca²⁺ and carbohydrate binding activities are limited to those CTLDs that contain appropriate residues at the principal calcium binding site (formed primarily from loop residues). Variations in the lengths and sequences of the loops determine the presence or absence of calcium binding sites.

C-type lectins bind carbohydrate in a calcium-dependent manner by direct ligation of two of the saccharide hydroxyls to the lectin bound calcium ion. The specificity of the carbohydrate recognition domain (CRD) for various sugars is determined by interactions of the carbohydrate with calcium-ligating residues and with residues surrounding the calcium binding site. Although the affinity for monosaccharides is typically weak (50 μM–10 mM), the overall strength of the interaction can be increased by two strategies. The binding affinity can be increased by engaging multiple glycan residues across an extended carbohydrate binding site (Feinberg et al., 2001; Somers et al., 2000), and lectin domain oligomerization can increase the avidity of the interaction with carbohydrate arrays.

CD23 binds to some of its ligands in a lectin-like manner by binding to their carbohydrates in a calcium-dependent fashion. CD23 binds to IgE in a non-lectin-like manner, however, as it can bind to deglycosylated IgE (Vercelli et al., 1989), indicating that IgE binding is

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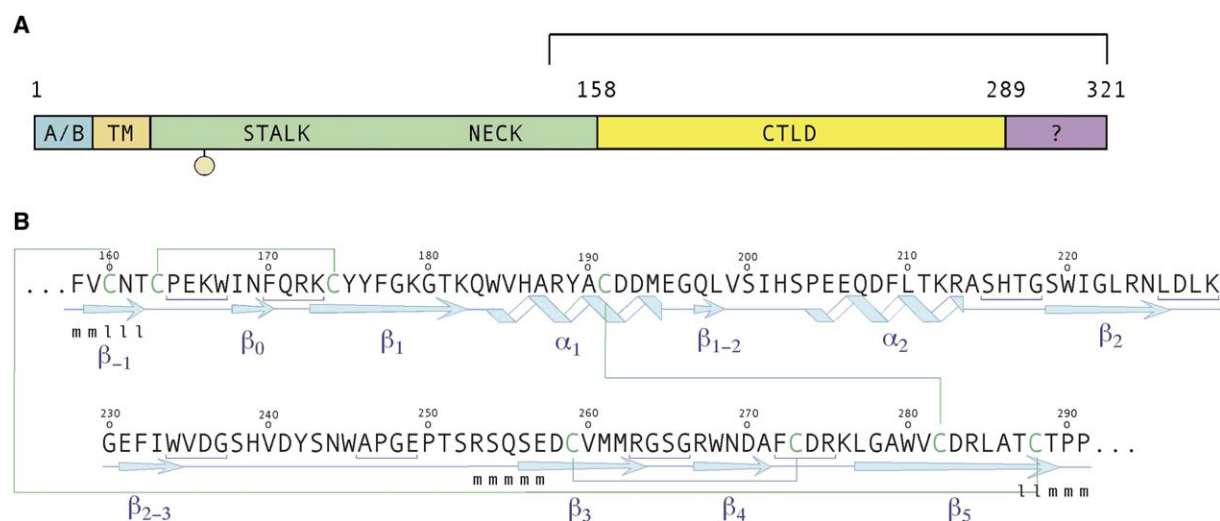


Figure 1. Domain Location, Sequence, and Secondary Structure of the CD23 CTLD

(A) Schematic of the domain organization of CD23. The short N-terminal cytoplasmic region (isoforms a and b) is shown in blue, the transmembrane region is shown in orange, the stalk and neck regions are shown in green, the CTL domain is shown in yellow, and the 30 C-terminal residues are shown in purple. The single N-linked glycosylation site (N63) is represented by a circle. The construct described in the text is indicated by the upper bracket.

(B) Sequence and secondary structure diagram of the apo CD23 CTLD. β strands are indicated by arrows, α helices are indicated by ribbons, and reverse turns are indicated by purple brackets. Disulfide bonds are shown with green lines. Where the secondary structure of the Ca^{2+} bound form differs from that of the apo form, the difference is noted below the diagram. "l" indicates nonregular secondary structure, and "m" means that these residues are not observed in the Ca^{2+} bound structure. There are two nonsilent mutations in the construct: H213R and G256S.

mediated by protein-protein contacts. While CD23 binds to IgE in a carbohydrate-independent manner, the binding is still calcium dependent (Richards and Katz, 1990). In addition, the binding site on the IgE-Fc for CD23 is thought to be different from the site for Fc ϵ RI (Garman et al., 2000; Wurzburg and Jardetzky, 2002) and may involve a unique conformational state of the IgE-Fc (Wurzburg et al., 2000; Wurzburg and Jardetzky, 2002).

The CD23 binding site on IgE has been mapped to the outer face of the C ϵ 3 domain (Chretien et al., 1988; Ghaderi and Stanworth, 1993; Sayers et al., 2004; Vercelli et al., 1989), and the oligomeric state of CD23 significantly affects IgE binding affinity. Each IgE-Fc dimer contains two potential CD23 binding sites, and it has been demonstrated that two CD23 lectin domains bind to one IgE-Fc (Shi et al., 1997). The native CD23 structure is thought to be trimeric, with a binding affinity of $\sim 10^{-8}\text{M}$ (Chen et al., 2002; Dierks et al., 1993; Kelly et al., 1998). Oligomeric CD23 binds IgE with a dual affinity, with the lower-affinity component attributed to the binding of a single lectin domain, and the higher-affinity component attributed to the binding of a second lectin domain (Chen et al., 2002). Monomeric CD23 lectin domains bind to the IgE-Fc with substantially lower affinity ($K_d = \sim 10^{-5}\text{M}$) than oligomeric CD23 (Chen et al., 2002; Shi et al., 1997).

Oligomerization of the CD23 lectin domain is not sufficient for high-affinity binding, however, as substitution of the CD23 stalk with stalk regions from other proteins results in the loss of high-affinity binding (Chen et al., 2002; Kelly et al., 1998). When the entire ectodomain of CD23 is fused to a heterologous trimerization domain, high-affinity binding is observed (Chen et al., 2002; Kelly et al., 1998); deletion analysis of the native stalk has implicated a region near the lectin domain (the "neck") as

necessary for this high-affinity interaction (Chen et al., 2002). While the stalk region enhances CD23 oligomerization, the neck region appears to be less tightly associated, potentially allowing for some flexibility in orienting the lectin domains to bind to IgE, enabling the high-affinity interaction (Kilmon et al., 2004). While CD23 oligomerizes on cell surfaces (Kilmon et al., 2004), the protein is naturally susceptible to endogenous proteases, and cleavage from the membrane and additional proteolysis release soluble fragments (37 kDa, 33 kDa, 29 kDa, 25 kDa, and 16 kDa) that contain the lectin domain and have been reported to have various cytokine activities (Armant et al., 1995; Hermann et al., 1999; Lecoanet-Henchoz et al., 1997).

We have solved the crystal structures of the soluble C-terminal fragment of CD23 in the presence and absence of Ca^{2+} . Calcium binding is observed at the principal binding site, but not at an auxiliary site, in contrast to the conclusions of a recent NMR study of CD23 (Hibbert et al., 2005). In the crystal structure of the apo form, the principal calcium binding site is occupied by an arginine from a neighboring loop, while in the calcium bound form, this arginine and the surrounding loop residues are disordered. Differences in the CD23 structure associated with calcium binding overlap with regions previously identified as important for IgE-Fc interactions (Bettler et al., 1992), suggesting that localized changes surrounding the principal calcium binding site affect the affinity for IgE.

Results and Discussion

A fragment of human CD23 (sCD23), including residues 150–321 (Figure 1), was expressed in insect cells by using the baculovirus system and was purified to

Table 1. Data Collection and Refinement Statistics

Space Group (Crystal)	$P2_12_12_1$ (Ca ²⁺)	C2 (apo, cryo)	C2 (apo, RT)
Unit cell dimensions			
a (Å)	40.31	112.4	113.2
b (Å)	51.26	50.7	51.6
c (Å)	58.77	75.1	75.8
β (°)		127.1	126.7
Molecules/asymmetric unit	1	2	2
Resolution ^a (Å)	30–1.3 (1.32–1.30)	30–1.5 (1.66–1.50)	30–2.75 (2.85–2.75)
Completeness ^a	99.4% (100%)	99.5% (99.8%)	99.6% (100%)
Reflections, unique (total)	30,523 (214,231)	53,947 (353,285)	9,254 (34,320)
Average redundancy	>7	7	3.7
$\langle I/\sigma_I \rangle$	41.6	18.7	9.6
R_{sym}^b (%) ^a	9.0 (33.8)	5.1 (28.1)	14.4 (45.2)
Number of reflections (free)	28,870 (1,561)	53,947 (2,727)	9,254 (480)
$R_{\text{cryst}}/R_{\text{free}}^c$ (%)	16.9/20.2	12.9/17.0	25.7/28.1
Atoms, total	1,173	2,535	2,169
Protein	999	2,169	2,090
Water	173	366	79
Calcium ion	1	0	0
Average B factor (Å ²)			
Protein	17.7	22.9	31.2
Water	32.5	39.4	35.0
Calcium ion/water ligands	15.7/25.6	—	—
Rms deviations from ideality			
Bond angles	1.497°	1.376°	1.093°
Bond lengths	0.020 Å	0.022 Å	0.004 Å
Ramachandran plot			
Most favored regions (%)	91.4	89.7	81.6
Allowed regions (%)	5.7	9.1	17.5
Generous regions (%)	2.9	1.3	0.9
Disallowed (%)	0.0	0.0	0.0

^aValues for the highest-resolution shell are shown in parentheses.

^b $R_{\text{sym}} = \sum_h \sum_i (|I_i(h)| - \langle I(h) \rangle) / \sum_h \sum_i I_i(h)$, where $I_i(h)$ = the observed intensity and $\langle I(h) \rangle$ = the mean intensity for multiple measurements.

^c R_{cryst} and $R_{\text{free}} = \sum_h |F(h)_o| - |F(h)_c| / \sum_h |F(h)_o|$ for the working and test set reflections, respectively.

homogeneity. sCD23 was crystallized in the presence and absence of Ca²⁺. Apo sCD23 crystals diffracted X-rays to a resolution of 1.5 Å, while the Ca²⁺-containing sCD23 crystals diffracted X-rays to 1.3 Å resolution (Table 1). The apo crystals grew in space group C2 and contained two monomers per asymmetric unit, while the Ca²⁺ bound crystals grew in space group $P2_12_12_1$ and contained a single monomer per asymmetric unit. The structure of apo sCD23 was solved by molecular replacement by using a model based on the asialoglycoprotein receptor. The model was refined and subsequently used to solve the crystal structure of the Ca²⁺ bound sCD23. Models were refined to an overall R_{factor} (R_{free}) of 12.9% (17%) for the apo sCD23 and 16.9% (20.2%) for the Ca²⁺ bound sCD23 (Table 1).

Description of the Apo CD23 Structure

The secondary structure and disulfide map of the CD23 lectin domain are shown in Figure 1. The core is very similar to other CTLs and has $C\alpha$ rms deviations ranging from 0.97 to 1.53 Å (over 106–118 $C\alpha$ pairs) when compared to the asialoglycoprotein receptor, mannose binding protein, and DC-SIGN. In addition to the five major β strands, two α helices, and two disulfides common to CTLs, the CD23 lectin domain contains two additional β strands at the N terminus (β_{-1} and β_0), two short β strand regions within the domain (β_{1-2} and β_{2-3}), two additional disulfides, and eight tight turns (Figure 2A). The model for apo sCD23 starts at residue F158 (Figures 1 and 2) and proceeds into a short β strand (β_{-1}). The first

cysteine of the domain (residue 160) forms a disulfide with C288, linking the beginning and end of the lectin domain. This additional disulfide is not conserved but is present in some family members such as DC-SIGN. A second cysteine (C163) forms a disulfide between C163 and C174, classifying CD23 as a member of the long-form CTLDs. Both α helices in CD23 end with short stretches of 3_{10} helix and are more similar in length to the helices in MBP than to those of the more closely related DC-SIGN. Reverse turn 4 changes conformation in the Ca²⁺ bound CD23 (Figure 2B), and residues 226–231 (including turn 4) will be referred to as Loop 1. The last β strand of the domain (β_5) is unusually long and twisted and continues almost to the end of the structure. Electron density for the 30 C-terminal residues (292–321) of the construct is not observed, and these residues are not part of the predicted lectin domain.

Structural Changes in the Ca²⁺ Bound CD23

The core of the lectin domain of the Ca²⁺ bound CD23 remains almost unchanged compared to that of the apo CD23: the average rms deviation over 118 $C\alpha$ atom pairs is only 0.30 Å (0.29 Å over all atoms, 904 pairs). However, the structure of the Ca²⁺ bound CD23 lectin domain differs significantly in three regions: the calcium binding site, the Loops 1 and 4 region, and the N and C termini (Figure 2B). Residues that act as calcium ion ligands become reordered upon calcium binding, and most of the changes are in the side chain orientations (Figure 3). One residue that sometimes changes in Ca²⁺ versus

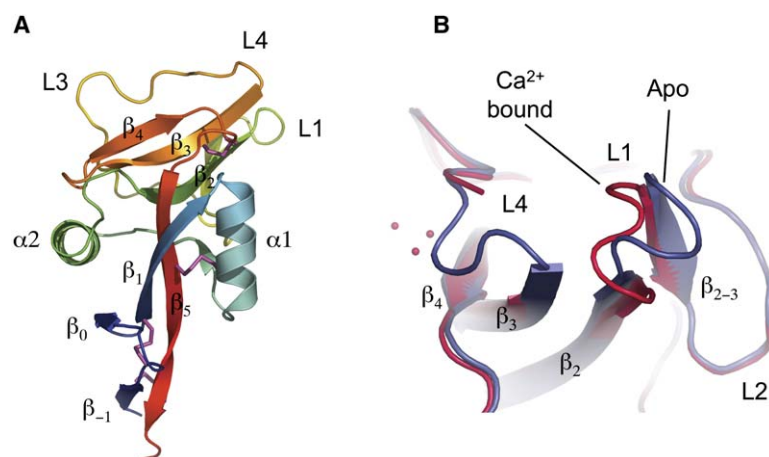


Figure 2. Structure of the CD23 CTLD

(A) A ribbon diagram of the apo CD23 CTLD shown colored from blue (N terminus) to red (C terminus). The canonical β strands are labeled 1–5 and are shown as arrows, the α helices are shown as ribbons, and Loops 1, 3, and 4 are indicated. Disulfide bonds are shown in magenta. The observed N and C termini of the Ca^{2+} bound CD23 are slightly shorter, and residues 253–257 are missing. (B) A ribbon diagram overlay of the apo and Ca^{2+} bound CD23 structures at the Loops 1 and 4 regions. The view is rotated $\sim 90^\circ$ from the orientation in (A). The apo form is shown in blue, and the Ca^{2+} bound form is shown in red; bound calcium and water ligands are shown as spheres. Loop 4 is missing from the Ca^{2+} bound form, while the conformation of Loop 1 changes significantly.

apo forms of CTLs is the conserved binding site proline (P250 in CD23). In Ca^{2+} bound MBP structures, this proline is always observed in the *cis* conformation, but in apo structures it has been observed in both the *cis* and *trans* conformations (Ng et al., 1998). In CD23, P250 is observed in the *cis* conformation in both the Ca^{2+} bound (one chain) and apo forms (two chains).

Upon calcium binding, the conformation and flexibility of Loops 1 and 4 change significantly (Figure 2B). Loop 4 is adjacent to the Ca^{2+} binding site on one side and packs next to Loop 1 on the other side. In the apo form, Loop 4 (residues 253–257) is ordered, and the side chain of R253 points into the Ca^{2+} binding site. When calcium binds, it replaces the R253 side chain in the binding site, and Loop 4 residues 253–257 become disordered (Figure 2B). Loop 1 residues (including turn 4) are affected by the increased flexibility of Loop 4. The main chain path changes, moving Loop 1 toward Loop 4 and converting reverse turn 4 from a type I to a type III' turn. The change in conformation at this turn is most evident in the movement of residue L228, which points away from Loop 4 in the apo form but points toward Loop 4 and moves 5.4 Å closer to it in the Ca^{2+} bound form. Except for the N and C termini, modeled residues in these loops have higher temperature factors than the rest of the structure, with

average B factors of 41 Å^2 and 30 Å^2 , respectively, compared to an overall B factor of $18\text{--}23 \text{ Å}^2$ (in the two forms).

The observed N and C termini of the protein are truncated in the Ca^{2+} bound form, with residues 158–159 and 289–291 missing from the Ca^{2+} bound structure. The effect on the secondary structure is the loss of the β_{-1} strand and the shortening of the β_5 strand (Figure 1). These changes are distant from the calcium binding site.

The Calcium Binding Site

The crystal structure of the lectin domain of MBP-A revealed three calcium ion binding sites (Weis et al., 1992): the principal site (site 2) conserved among CTLs where carbohydrate binding occurs, an auxiliary site (site 1) found in many but not all CTLs, and a third site (site 3) that was most likely due to the crystallization conditions. Unlike MBP and DC-SIGN, human CD23 contains only the principal Ca^{2+} binding site. The auxiliary calcium site in MBP is formed by carboxylate-containing residues from Loops 1 and 4—D189, E193, D216, and D222. Proteins lacking the auxiliary calcium binding site have shortened loop regions and/or lack appropriate residues for calcium ligation. Of the four carboxylate ligands in MBP, only one is conserved in CD23 (N225,

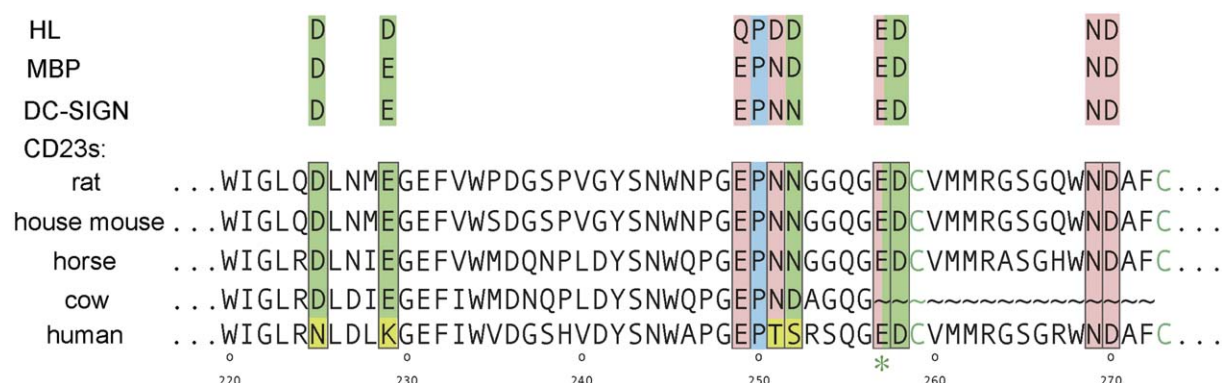


Figure 3. CD23 Principal and Auxiliary Calcium Site Ligands in Various Species

An alignment of CD23 sequences from rat, house mouse, horse, cow (partial), and human sources showing the calcium-ligating residues. For comparison, the corresponding residues from the human asialoglycoprotein receptor (HL), rat MBP-A, and human DC-SIGN are shown above the CD23 sequences. Principal site ligands are highlighted in pink, the conserved *cis* proline is highlighted in blue, and auxiliary site ligands are highlighted in green. Nonconservative substitutions in the human sequence are highlighted in yellow. The glutamate marked with an asterisk acts as both a principal (side chain O_ϵ) and auxiliary (main chain O) site ligand. The human CD23 numbering is given below the sequences.

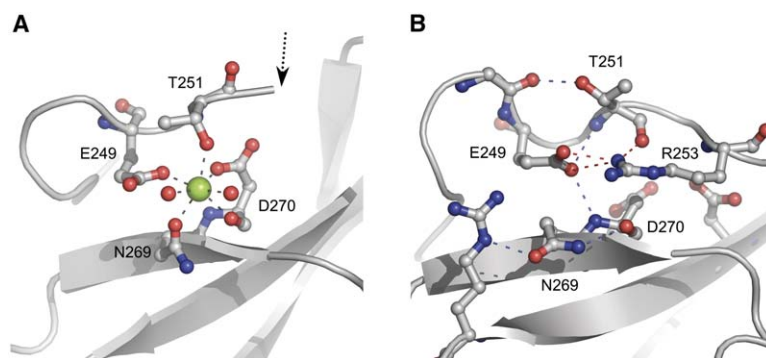


Figure 4. The Calcium Binding Site

(A) The Ca^{2+} bound CTLD. The calcium is shown as a green sphere, and its ligands come from residues E249 ($\text{O}\epsilon 1$), T251 ($\text{O}\delta 1$), N ($\text{O}\delta 1$), and D270 ($\text{O}\delta 2$ and O) and two water molecules (red spheres). Ligand bonds are indicated with dashes, and the beginning of the missing Loop 4 residues (253–257) is indicated with an arrow. E249, P250, and T251 form the “EPT” motif discussed in the text. (B) The Ca^{2+} binding site in the apo form, with the guanidinium group of R253 occupying the former calcium ion position. Ionic and hydrogen bonds formed by the guanidinium group are indicated with red dashes. Residues that form new contacts with the calcium-ligating residues are also shown; hydrogen bonds are indicated by blue dashes.

K229, S252, D258) (Figure 3). Although N225 $\text{O}\delta 1$ and the S252 $\text{O}\gamma$ might serve as calcium ligands, the orientation of the potential ligands is not appropriate.

The principal site calcium ion in MBP usually has eight coordinating ligands; six ligand atoms are contributed by the protein and the remaining ligands are contributed either by two water molecules or by two vicinal hydroxyls from bound carbohydrate (Ng et al., 1998; Weis et al., 1992). The six protein ligands are oxygen atoms, either side chain or main chain, from residues such as Asp, Asn, Glu, and Gln. A 3 residue sequence motif, containing the conserved *cis* proline (P250 in CD23), determines the sugar binding specificity. The “EPN” motif is found in lectins that preferentially bind mannose-type sugars (vicinal 3- and 4-hydroxyls are equatorial), while the “QPD” motif is found in lectins that preferentially bind galactose-type sugars (3-hydroxyl is equatorial, while the 4-hydroxyl is axial). The calcium-ligating residues bind not only to the calcium ion, but they also make key contacts to the carbohydrate. The intimate contacts linking the calcium ion to its protein ligands and the same protein residues to the carbohydrate are essential for the stability of the ternary complex. For example, when the MBP-A site was mutated from EPN to EPD, the oxygen ligand to the calcium ion was preserved, but the hydrogen bond donor for the sugar-OH group was lost. This mutant MBP-A bound calcium but was unable to bind carbohydrate (Weis et al., 1992).

In CD23, the calcium ion is heptavalently coordinated to atoms from four protein residues, E249 ($\text{O}\epsilon 1$), T251 ($\text{O}\gamma 1$), N269 ($\text{O}\delta 1$), and D270 ($\text{O}\delta 2$ and O), and two water molecules (Figures 4 and 5). The resulting geometry of the calcium coordination is square planar bipyramidal instead of a pentagonal bipyramidal. Three features of this site are unusual. First, the motif at the preference-determining residues is “EPT” (residues 249–251), not “EPN” or “QPD.” The threonine hydroxyl group may be able to contribute both an oxygen lone pair to the calcium and form a hydrogen bond to a carbohydrate hydroxyl, maintaining both calcium and sugar binding capabilities, but the geometry of the interactions will not be the same as that with an Asn residue. The tunicate lectin TC14 has a serine at this position that fails to act as a calcium ligand but binds carbohydrate through a water molecule (Poget et al., 1999). The Thr side chain, while bulkier than that of Ser, is shorter than an Asn or Asp

side chain, and therefore a sugar moiety might have to move deeper into the site to bind to the Thr.

The second unusual feature of the CD23 Ca^{2+} binding site is that Loop 4 is disordered (residues 253–257 are not observed). While disorder has been observed at a binding site loop in an apo CTLD structure (Ng et al., 1998), disorder in a calcium bound form is unusual and suggests that Loop 4 could adopt distinct conformations in different CD23 complexes with either IgE and/or carbohydrate.

The third unusual feature of this site is that residue E257 (MBP-A E193 equivalent) was expected to contribute a calcium ligand; however, electron density was not observed for this residue. It is the last of the Loop 4 residues (R-S-Q-S-E) missing from the structure. In human P-selectin, the equivalent residue (E88) is swung out of the site in the Ca^{2+} bound form and cannot contribute a ligand for the calcium (Graves et al., 1994; Somers et al., 2000); however, binding site alterations are observed upon carbohydrate binding. In E-selectin, the side chain of N83 rotates so that it can act as a calcium ligand and bind to the sugar moiety through a water molecule. In P-selectin, a more dramatic change is observed upon binding a glycopeptide. E88 moves into the site to coordinate the calcium ion and to bind the carbohydrate directly (Somers et al., 2000). The return to the binding site is accomplished by a large movement of the loop containing E88, which is unusual in that the carbohydrate binding site is usually “preformed” in the presence of calcium and significant changes upon carbohydrate binding are not expected. It is possible that carbohydrate binding to CD23 will induce a change in Loop 4, bringing E257 into the calcium coordination sphere. The change would be quite dramatic, however, as the carboxylate oxygens of E257 are ~16 Å away from the calcium binding site in the apo form. A neighboring residue, D258, might be the preferred ligand as its carboxylate oxygens are only ~6 Å away from the calcium site in both the apo and Ca^{2+} bound forms, and a smaller change in Loop 4 would be needed to bring this residue closer to the calcium ion.

Apo CD23

Typically, apo CTLs are less “rigid” than their Ca^{2+} bound forms—Loop 4 (adjacent to the Ca^{2+} binding site) has been observed in multiple conformations in MBP (Ng

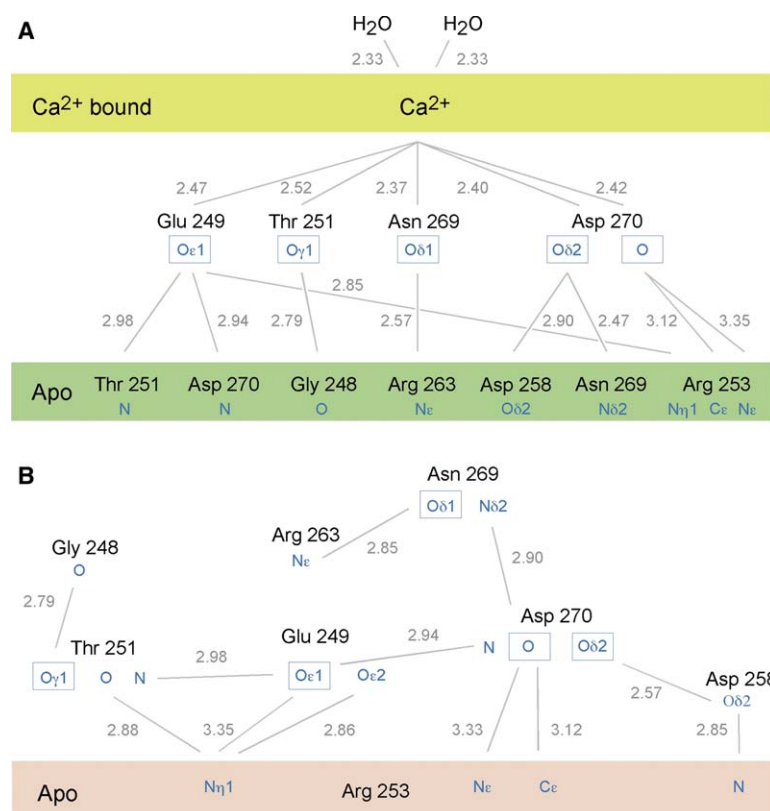


Figure 5. Interactions at the Calcium Binding Site and the Role of Arg-253

(A) The calcium ion is heptavalently coordinated in CD23 (distances in Å). Four CD23 residues (E249, T251, N269, and D270) contribute five ligands (boxed in blue) for the calcium ion. Two water molecules occupy the remaining ligand positions. In the absence of Ca²⁺, the residues in the binding site rearrange, and the former calcium ligands form alternate interactions with other CD23 atoms (shown in green).

(B) The role of R253. In addition to the rearrangement of the calcium binding residues in the apo form, R253 moves into the Ca²⁺ binding site, and its guanidinium group occupies the former calcium ion position. Atoms from R253 interact directly with 3 of the 4 residues that normally act as Ca²⁺ ligands, and former calcium ligating residues form new interactions.

et al., 1998), and apo CTLs are susceptible to protease cleavage. Unusually, apo CD23 is more structured in the crystals than in the Ca²⁺ bound form, with all residues of Loop 4 well ordered and in essentially the same conformation in both apo protomers. Unlike other apo CTLs, the “empty” calcium binding site of CD23 is actually filled by the side chain of a Loop 4 residue, Arg-253, that has its N_η1 atom occupying the calcium ion position (Figure 4B). Arg-253 makes six contacts within the site (Figures 4B and 5), including contacts with three of the four calcium binding residues. In addition, every atom that acts as a ligand to the calcium ion forms a new interaction in the apo structure. The extensive contacts that Arg-253 makes in the apo form probably help to stabilize Loop 4 in its observed conformation. Since Loop 4 would be expected to be more mobile and exposed in the Ca²⁺ bound form of CD23, protease digestion experiments with apo and Ca²⁺ bound CD23 were carried out with trypsin, Glu-C, and subtilisin (data not shown). No evidence for enhanced proteolytic cleavage of the Ca²⁺ bound CD23 was obtained. However, the disordered region in Loop 4 is sufficiently short (5 residues) that it may not be accessible to proteases.

Comparison of the NMR and Crystal Structures of the CD23 Lectin Domain

The structure of derCD23 (residues 156–298) was recently determined by NMR (Hibbert et al., 2005). The region surrounding the principal calcium binding site (residues 245–258) is poorly defined in the NMR structures (Figure 6A). In contrast, the crystal structures are well defined in this region (except for residues 253–257, which are not observed in the Ca²⁺ form) and have es-

entially the same backbone conformation in both the apo and Ca²⁺ bound forms. None of the NMR structures contain a geometrically competent principal calcium binding site. This may be due, in part, to the modeling of the conserved Pro-250 of the EPT motif as the *trans* instead of the *cis* isomer observed in all calcium bound CTLs (Figure 6B). Both the apo and Ca²⁺-containing crystal structures have geometrically competent principal calcium binding sites (occupied in the apo form by the guanidinium group of R253).

In the NMR experiments, chemical shift changes observed upon calcium binding were also attributed to the binding of a calcium ion at the putative auxiliary site rather than at the principal site. In contrast, the crystal structure clearly shows calcium bound at the principal binding site (14σ electron density peak), while no extra electron density is observed in the region of the potential auxiliary binding site. A sequence alignment of CD23 from horse, cow (partial sequence), mouse, rat, and human sources shows that the principal calcium binding site is preserved across all species (Figure 3), although it is slightly altered in the human sequence (N251T). CTLs that have an auxiliary binding site have either three or four carboxylate side chain ligands for the calcium ion. The auxiliary site is conserved in equine, bovine, murine, and rat CD23 sequences, but not in human CD23; only one of the carboxylate ligands is present. In the crystal structures, the potential auxiliary site residues are not oriented appropriately to ligate a calcium ion. It is unlikely that the auxiliary calcium binding site exists in human CD23.

Of the 8 residues experiencing significant ¹H chemical shifts in the presence of calcium in the NMR

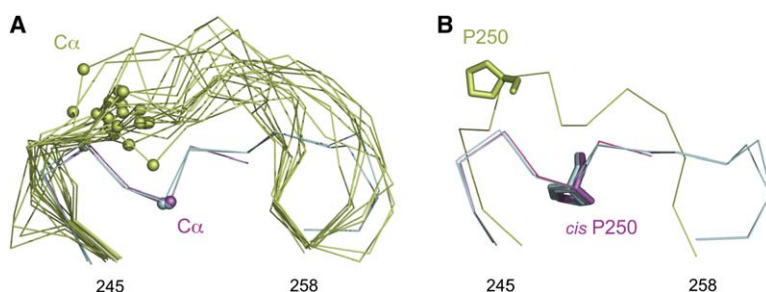


Figure 6. Comparison of Residues 245–258 from the NMR and Crystal Structures

(A) An overlay of the main chains from the NMR structures (1T8C, shown in green) with the apo (blue) and calcium bound (magenta) CD23 from the crystal structures. The $C\alpha$ atoms of P250 are shown as spheres for each model.

(B) A comparison of the positions of the conserved P250. This proline is modeled as the *trans* isomer in all of the NMR structures (model 1 shown), but it is modeled as the *cis* isomer in the crystal structures.

experiments, 4 were in or near Loop 1 (N225, K229, G230, and E231), and 3 were in or near the principal calcium binding site (D270, A271, D274). The crystallographic observation of a large change in the position of Loop 1 upon calcium binding is consistent with these NMR data. Residues 252–257 of Loop 4 were not assigned in the NMR structures, and therefore changes in this loop upon calcium binding could not be observed. The fact that these residues could not be assigned is also consistent with the observation that these residues are disordered in the calcium-containing crystal structure. The assumption that the largest changes in chemical shift identify the location of calcium binding is not borne out by the crystal structures.

Finally, a single core packing arrangement is observed for both the apo and Ca^{2+} bound CD23 crystal structures, and this arrangement differs from the two core packing arrangements observed in the NMR structures (database entries 1T8C and 1T8D).

The Carbohydrate Binding Site

Carbohydrates bind at the principal calcium binding site of CTLs, with the ligand preference determined by the 3 residue motif that includes the conserved *cis* proline. Preferential binding to mannose-like sugars is observed in lectins containing the “EPN” motif, while those lectins containing the “QPD” motif preferentially bind galactose. Murine CD23 has the “EPN” motif, suggesting that it preferentially binds mannose-type carbohydrate, while human CD23 has “EPT” at the binding site. CD23 has been reported to bind to both mannose-type carbohydrates (Pochon et al., 1992; Richards and Katz, 1990) and galactose-containing carbohydrates (Kijimoto-Ochiai, 2002; Kijimoto-Ochiai and Uede, 1995; Richards and Katz, 1990). We modeled N-acetylglucosamine, mannose, and galactose into the principal calcium binding site of the Ca^{2+} bound CD23. Each of the sugars could be accommodated in the site; however, the hydrophobic face of galactose makes fewer van der Waals contacts in the CD23 site than it does in the TC14 lectin structure because of the substitution of a key tryptophan residue for arginine in CD23. There are no steric hindrances in the current structure that would prevent any of these sugar moieties from binding. However, because 5 residues of Loop 4 near the calcium binding site are not observed in the Ca^{2+} bound CD23 structure (including expected Ca^{2+} ligand E257), we cannot be certain that the site will not change upon carbohydrate binding.

Larger oligosaccharides can make additional contacts to the lectin outside the principal calcium binding site, as illustrated by the structures of DC-SIGN bound to oligo-

saccharides (Feinberg et al., 2001; Guo et al., 2004). Additional binding sites are likely to be very specific to each lectin, depending on its sequence and surface topology. For example, in DC-SIGN, a carbohydrate binding pocket is created by a “gap” between the end of the $\alpha 2$ helix and an adjacent turn. The position of the $\alpha 2$ helix is one of the more variable structural elements in the CTLDs. In CD23, the main chain paths of the helix and turn close the gap, eliminating this pocket. At another distal site, 3 residues in DC-SIGN (D367, L371, and K373) form part of a hydrogen bonding network that accommodates binding of a sugar moiety. In CD23, the structurally homologous residues L277, A271, and M361 instead form a hydrophobic interaction. In addition to forming additional carbohydrate binding sites, surface residues can be used to discriminate between carbohydrate linkages. In DC-SIGN, F313 binds to distal carbohydrate moieties that have high mannose linkages while excluding complex N-linked glycans. Although F313 is part of a 2 residue insertion relative to CD23, two arginine guanidinium groups point toward each other at approximately the same location in CD23, forming a “hump” on the protein surface.

Implications for IgE Binding

The IgE binding site was first identified as being confined to the lectin domain of CD23 (Bettler et al., 1989), and homology scanning mutagenesis and monoclonal antibody binding further identified six short segments important for IgE binding to two regions between residues 165–190 and 224–256 (Bettler et al., 1992). While mutagenesis alone could not distinguish between substitutions that directly affected IgE binding from those that affected the conformation of the protein, monoclonal antibody binding results suggested that changes in segments 165–173 and 176–183 affected the global conformation of the protein. These segments contain the $\beta 0$ strand and the two tight turns bracketing it as well as part of the $\beta 1$ strand. The $\beta 0$ strand may form part of the neck region that is necessary for the correct orientation of the lectin domains for optimal IgE binding (Kilmon et al., 2004), while the $\beta 1$ strand forms hydrogen bonds with the $\beta 0$ and the $\beta 5$ strands. The remaining four segments were determined to disrupt IgE binding only. Segment 185–190 is from the N-terminal half of the $\alpha 1$ helix. Segment 224–233 contains Loop 1, whose main chain path changes dramatically upon calcium binding (Figure 2B). Segment 238–243 contains part of Loop 2, and segment 251–256 contains one of the calcium-ligating residues and most of Loop 4, which becomes disordered upon calcium binding. The four segments define

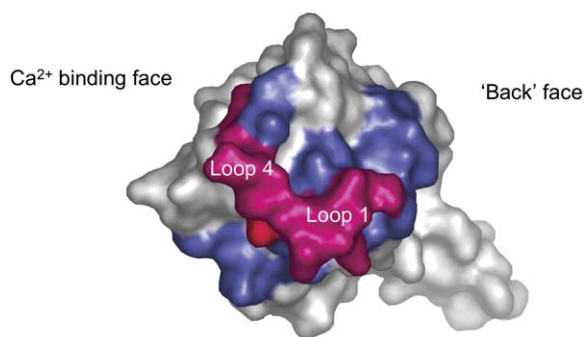


Figure 7. A Surface Representation of the CD23 CTLD

Residues implicated by mutagenesis studies as being important for IgE binding are shown in blue, residues whose positions change from the apo to the Ca^{2+} bound form are shown in red, and the overlap of these residues is shown in magenta.

a surface encompassing the upper, far right side of the domain's carbohydrate binding face and continuing around the top "back side" of the domain (Figure 7). In addition, residues from Loops 1, 2, and 4 surround a hydrophobic pocket on the back face of the lectin. The crystal structure of CD23 shows that residues in segment 224–233 (Loop 1 region) contact adjacent residues from the other three segments (185–190, 238–243, and 251–256), suggesting that Loop 1 residues form the core of the IgE binding surface. Changes in the other segments might affect the position of Loop 1 residues and thereby alter IgE binding. Although Loop 1 is removed from the calcium/carbohydrate binding site, the conformations of Loops 1 and 4 change upon calcium binding, offering an explanation for the calcium-dependent, but carbohydrate-independent, binding of IgE to CD23.

Calcium may play a purely structural role in IgE binding by affecting the CD23 conformation, as suggested by the crystal structures, or, alternatively, it may participate directly in the protein:protein interaction interface. The transition of Loop 4 from ordered (apo form) to disordered (calcium bound form) may be linked to the apparently contradictory requirement for calcium in a carbohydrate-independent interaction. If residues required for IgE-Fc binding are locked in a nonbinding or disfavored conformation in the apo form, the binding of calcium (and subsequent disordering of Loop 4) might liberate these residues to assume an IgE binding-competent conformation. While R253 is not conserved in other CD23 sequences, these proteins do apparently contain the auxiliary calcium binding site, which could also similarly influence Loop 4 and Loop 1 conformations in a calcium-dependent manner. Given that some CD23 isoforms are involved in transporting IgE complexes (Ewart et al., 2002; Montagnac et al., 2005; Yokota et al., 1992), it seems possible that calcium-dependent IgE binding could also provide a means of regulating IgE binding and release in a physiological setting.

Experimental Procedures

Protein Expression and Purification

The full-length human CD23 gene was obtained from Invitrogen (GeneStorm Clone H-M15059M). The clone contained two nonsilent sequence changes (H213R and G256S). R213 occurs at the end of the $\alpha 2$ helix and is distant from both the principal and auxiliary cal-

cium binding sites and from Loops 1 and 4. S256 occurs in the middle of Loop 4, but it does not appear to have diminished the flexibility of this loop significantly since this loop is disordered in the calcium bound form. A construct containing the lectin domain of the mature CD23 (residues 150–321) was generated by PCR and subcloned into the baculovirus transfer/expression vector pBACgus-3 (Novagen). This construct will be referred to as soluble CD23 or sCD23. Recombinant baculovirus was generated with the Baculogold system (Pharmingen) by using standard methods. Sf9 and Sf+ cells (Protein Sciences) were used to produce viral stocks.

sCD23 was expressed in Sf+ cells grown in shaker flasks in HyQ SFX insect medium (HyClone) to 1.5×10^6 cells/ml and infected with high-titer virus stock. Three days postinfection, supernatants were harvested, filtered, treated with a protease inhibitor cocktail, dialyzed against 6 volumes of 50 mM sodium phosphate, 300 mM sodium chloride (pH 7.0) at 4°C, and purified with Talon metal affinity resin (CLONTECH Laboratories, Inc.). Approximately 12 mg protein were obtained from 2 liters of supernatant. The N-terminal S and His tags were removed from the fusion protein by enterokinase digestion, which was confirmed by SDS-PAGE and by Western blotting with anti-His (Novagen) and anti-CD23 monoclonal antibodies (Santa Cruz Biotechnology, Inc.). The resulting sCD23 protein contains three additional vector-derived residues (S-P-G) at the N terminus. sCD23 was further purified by using gel filtration (Superdex 75) and anion exchange chromatography (Q2). Purified, recombinant sCD23 has a calculated molecular weight of 19,500, but it migrates on SDS-PAGE with an apparent molecular weight of 24,000.

Crystal Growth and Treatment

Crystals of sCD23 were initially obtained from a mixture (6.5:1 molar ratio) of sCD23 and IgE-Fc C ϵ 3-4 protein (Wurzburg et al., 2000), but they were subsequently reproduced with sCD23 alone. Crystals were grown by vapor diffusion by using the hanging drop method. Protein (10 mg/ml in 10 mM Tris [pH 8.0]) was mixed 1:1 with precipitant (1.2 M sodium monobasic phosphate, 0.8 M potassium dibasic phosphate, 0.1 M Caps buffer [pH 10.5], and 0.2 M lithium sulfate; final pH 6.2) and incubated at room temperature. Crystals were harvested into 1.3 M sodium phosphate, 0.7 M potassium phosphate, 0.1 M Caps buffer (pH 10.4), and 0.2 M lithium sulfate (final pH 6.2). Crystals used for cryo-data collection were transferred from harvest buffer to Paratone oil and flash cooled in liquid nitrogen.

sCD23 was also crystallized with Ca^{2+} in the presence of IgE-Fc C ϵ 3-4 (2:1 molar ratio). Protein (10 mg/ml in 30 mM Tris [pH 8.0], 10 mM CaCl_2) was mixed 1:1 with precipitant (17% [w/v] polyethylene glycol 4000, 100 mM Mes buffer [pH 6.0]) and incubated at room temperature. A single crystal was harvested into 21% polyethylene glycol 4000, 100 mM MES [pH 6.0], 5 mM CaCl_2 , transferred into Paratone oil, and flash cooled in liquid nitrogen.

Data Collection and Processing

Both apo CD23 crystals belong to space group C2 and have similar cell dimensions (Table 1). Data were collected from the room temperature crystal to 2.75 Å by using a rotating anode source ($\lambda = 1.54$ Å) and a Mar 345 Image Plate detector, and processed by using the HKL suite of programs (Otwinowski and Minor, 1997). Data from the cryocooled crystal (-160°C) were collected at the Advanced Photon Source, DND-CAT 5-ID beamline ($\lambda = 0.97895$ Å) to 1.5 Å by using a MarMosaic detector and processed with XDS (Kabsch, 2001). Data from the Ca^{2+} -containing crystal were collected at -160°C to 1.3 Å at the Advanced Photon Source, COM-CAT beamline ($\lambda = 1.02336$ Å) by using a MarCCD detector (Table 1). The crystal belongs to space group $P2_12_12_1$ with unit cell dimensions $a = 40.31$ Å, $b = 51.26$ Å, $c = 58.77$ Å. The data were processed and integrated with the HKL suite (Otwinowski and Minor, 1997).

Structure Determination and Refinement

A model for CD23 was generated from the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor (Meier et al., 2000) (PDB entry 1DV8) by deletion and mutation of residues, yielding a 121 residue model for the 175 residue sCD23 (with 44 residues identical to those of the CD23). Molecular replacement with CNS (Brunger et al., 1998) with data from the room temperature crystal gave a solution with two CD23 molecules per asymmetric unit and a correlation coefficient of 33.1%. An R_{free} test set was generated by

using reflections from thin-resolution shells. The solution was refined with CNS (Brunger et al., 1998) with all data from 30 to 2.75 Å resolution and an $|F| > 0$. An anisotropic bulk solvent correction was applied. Noncrystallographic symmetry restraints did not improve the R_{free} and were not used during refinement. Slight differences in the main chain (rmsd over 118 C α pairs of 0.34 Å) and side chain positions were apparent. Several cycles of building with O (Jones et al., 1991) and refinement with CNS (Brunger et al., 1998) yielded a model with an R_{work} of 25.7% and an R_{free} of 28.1%. This model contained residues F158–P291 and 79 water molecules and was missing residues 226–229 in chain A, residues 227–229 in chain B, and 11 residues from the N terminus and 30 residues from the C terminus of both chains.

Chain B from the apo CD23 room temperature model was used to solve the apo cryocooled structure, yielding a molecular replacement solution with a correlation coefficient of 56.7%. The R_{free} test set was generated with reflections from thin-resolution shells. The model was rebuilt with O and refined with CNS with all data from 30 to 1.5 Å with $|F| > 0$ and with an anisotropic bulk solvent correction. Anisotropic B factor refinement, bond length and angle refinement with Refmac5 (CCP4, 1994) provided a final model with 366 water molecules, an R_{work} of 12.9%, and an R_{free} of 17.0%. The final model includes all residues from F158 to P291.

Chain B from the apo cryocooled structure was used to solve the Ca²⁺-containing structure. A single solution with a correlation coefficient of 57.9% was found by using CNS. An R_{free} test set was generated with reflections from thin-resolution shells. After initial minimization and B factor refinement, a simulated-annealing composite omit map showed a 14 σ peak at the Ca²⁺ binding site along with reordering of the residue side chains ligated to the calcium ion. The structure was rebuilt with O and refined with CNS. Anisotropic B factors were refined with Refmac5 (CCP4, 1994) in the final stages. The final model includes residues 160–288 of CD23, 1 calcium ion, and 173 water molecules. The model is missing 13 N-terminal residues, 33 C-terminal residues (289–321), and internal residues 253–257. Some weak electron density for residues 253–257 was observed but could not be modeled. The final model has an R_{work} of 16.9% and an R_{free} of 20.2%.

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Accession Numbers

Coordinates and structure factor amplitudes for the CD23 structures have been deposited in the Protein Data Bank (PDB ID codes [2H2R](#) and [2H2T](#)).