

Storage function of cartilage oligomeric matrix protein: the crystal structure of the coiled-coil domain in complex with vitamin D₃

Suat Özbek¹, Jürgen Engel and Jörg Stetefeld

Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

¹Corresponding author
e-mail: Suat.Oezbek@unibas.ch

The five-stranded coiled-coil domain of cartilage oligomeric matrix protein (COMPcc) forms a continuous axial pore with binding capacities for hydrophobic compounds, including prominent cell signalling molecules. Here, we report the X-ray structure of the COMPcc domain in complex with vitamin D₃ at 1.7 Å resolution. The COMPcc pentamer harbours two molecules of the steroid hormone precursor in a planar *s-trans* conformation of the conjugated triene, with the aliphatic tails lying along the molecule axis. A hydrophilic ring of five Gln54 side chains divides the channel into two hydrophobic compartments in which the bound vitamin D₃ pair is fixed in a head-to-head orientation. Vitamin D₃ binding induces a volumetric increase of the cavities of ~30% while the main chain distances of the pentamer are retained. This adaptation to the bulky ring systems of the ligands is accomplished by a rotamer re-orientation of β-branched side chains that form the knobs into holes of the coiled-coil structure. Compared with binding of vitamin D and retinoic acid by their classical receptors, COMP exerts a distinct mechanism of interaction mainly defined by the pattern of hydrophobic core residues.

Keywords: hydrophobic pore/vitamin D₃ storage/X-ray crystallography

Introduction

Cartilage oligomeric matrix protein (COMP) (Oldberg *et al.*, 1992) is a non-collagenous glycoprotein expressed predominantly in articular cartilage, tendon and ligament (Hedbom *et al.*, 1992; DiCesare *et al.*, 1994; Smith *et al.*, 1997; Müller *et al.*, 1998). It is a member of the thrombospondin (TSP) gene family of extracellular glycoproteins (Adams, 2001) and, like TSP-3 and TSP-4 (Mörgelin *et al.*, 1992; Qabar *et al.*, 1995; Shen *et al.*, 1995; Ekman *et al.*, 1997), COMP is assembled into a pentameric bouquet-like structure (Mörgelin *et al.*, 1992), which is stabilized by interchain disulfide bonds in the N-terminal coiled-coil domain (residues 20–83) (Efimov *et al.*, 1994; Malashkevich *et al.*, 1996). The other two known TSPs, TSP-1 and TSP-2, are assembled into three-stranded oligomers (O'Rourke *et al.*, 1992). All TSPs are characterized by a multidomain architecture, which in the case of COMP comprises the N-terminal heptad repeat

region (cc) followed by four epidermal growth factor (EGF)-like domains (EF), seven calcium-binding domains (T3) and a C-terminal globular domain (TC). Mutations in the COMP gene shown to affect the conformation of the calcium-binding domains are connected with hereditary skeletal disorders, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED), which involve short stature and joint laxity (Hecht *et al.*, 1995, 1998; Briggs *et al.*, 1998; Thur *et al.*, 2001). It has been shown recently that COMP-null mice do not exhibit any altered phenotype of skeletal development, indicating that the pentameric TSPs may be functionally redundant in the respective tissues and that PSACH and MED are not caused by a reduction of COMP in the extracellular matrix, but instead by the effects of a mutant COMP (Svensson *et al.*, 2002).

The seco steroid vitamin D₃ is the primary precursor needed for cartilage and bone mineralization, and the regulation of calcium and phosphate homeostasis (Bouillon *et al.*, 1995). It is produced in the skin via rupture of the 9,10 carbon bond of 7-dehydrocholesterol after exposure to sunlight. The active steroid hormone 1α,25(OH)₂D₃ is then generated by a sequential two-step metabolism of vitamin D₃ by the liver and kidney. Its classical function is the stimulation of transmembrane calcium transport in the intestine, bone and kidney. At the transcriptional level, the actions of 1α,25(OH)₂D₃ are mediated via high affinity binding ($K_D = 0.55$ nM) to the vitamin D nuclear receptor (VDR) (Rochel *et al.*, 2000). This complex forms a functional heterodimer with the retinoid X receptor and then induces the expression of target genes by binding to vitamin D-responsive elements (VDRE) (Haussler *et al.*, 1997). Serum transport of the essentially insoluble vitamin D₃ is facilitated by hydrophobic interaction with vitamin D-binding protein (VDB) (Ray, 1996; Verboven *et al.*, 2002). Several candidates, like annexin II (Baran *et al.*, 2000) and a putative 64.5 kDa protein (Nemere *et al.*, 2000b), have been proposed as membrane receptors for 1α,25(OH)₂D₃, but their characterization is as yet limited to immunochemical and functional studies. Recently, a novel VDR-independent activity of 1α,25(OH)₂D₃ has been defined that involves regulation of matrix metalloproteinase function via protein kinase C signalling (Maeda *et al.*, 2001). This process is believed to be mediated by rapid membrane-associated signalling, which results in extracellular matrix remodelling of growth plate cartilage. Interestingly, COMP expression in this tissue has been shown to be predominantly located in the proliferative region (Shen *et al.*, 1995; Ekman *et al.*, 1997), indicating a role for COMP in the developing cartilage.

The crystal structure of the recombinant coiled-coil domain of COMP (COMPcc) (Efimov *et al.*, 1996; Malashkevich *et al.*, 1996) revealed a 73 Å long hydrophobic channel with a diameter of 2–6 Å inside of the

Table I. Statistics for data collection and structure refinement

Data collection	
Resolution (Å)	1.7
Observed reflections	300.112
Unique reflections	22.133
Completeness	100
Redundancy	4.1
R_{sym}^a	5.9
Unit cell dimensions (Å, °)	
<i>a</i>	38.29
<i>b</i>	49.29
<i>c</i>	55.26
β	103.87
Refinement statistics	
R -factor ^b (%)	23.1
R_{free} (%)	25.4
Mean B -factor (Å ²)	
Protein	27.3
Water	41.5
Ligands I/II	55.9/71.8
Bonds (Å) ^c	0.005
Angle (°) ^c	0.9
Dihedrals (°) ^c	15.9
Improper (°) ^c	0.81

^a $R_{\text{sym}} = \sum I - \langle I \rangle / \sum I$.

^b R -factor = $\sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$.

^cRoot-mean-square error.

pentameric α -helical bundle. We could demonstrate binding of different hydrophobic compounds to the recombinantly expressed COMPcc, and have presented an X-ray structure of the COMPcc–all-*trans* retinol and COMPcc–benzene complexes (Guo *et al.*, 1998). This approach suggested a putative role for COMP as a storage and delivery protein for regulatory molecules in bone metabolism and led us to study in detail COMPcc–vitamin D₃ binding by determining the X-ray structure of this complex.

Our data present the coiled-coil domain of COMP as the first example of a structural motif with binding capacities for both vitamin A and D₃. A detailed structural comparison with the ligand binding domain (LBD) of the VDR and the vitamin A binding protein (CRBP) reveals novel features of protein–ligand interaction in the coiled-coil structure.

Results

Preparation and overall X-ray structure of the COMPcc–vitamin D₃ complex

The coiled-coil domain of COMP comprising residues 20–72 was obtained by recombinant expression in *Escherichia coli* as described previously (Efimov *et al.*, 1996). After purification in a totally reduced state, reoxidation to the disulfide-linked pentamer was facilitated by addition of oxidized and reduced glutathione at a 10:1 ratio. Pentamer formation of the coiled-coil domain is independent of disulfide linkage, and renatured COMPcc, irrespective of its oxidative state, shows a single species in native PAGE and in sedimentation experiments using analytical ultracentrifugation. After an incubation period of several weeks, the oxidative state of the COMPcc was

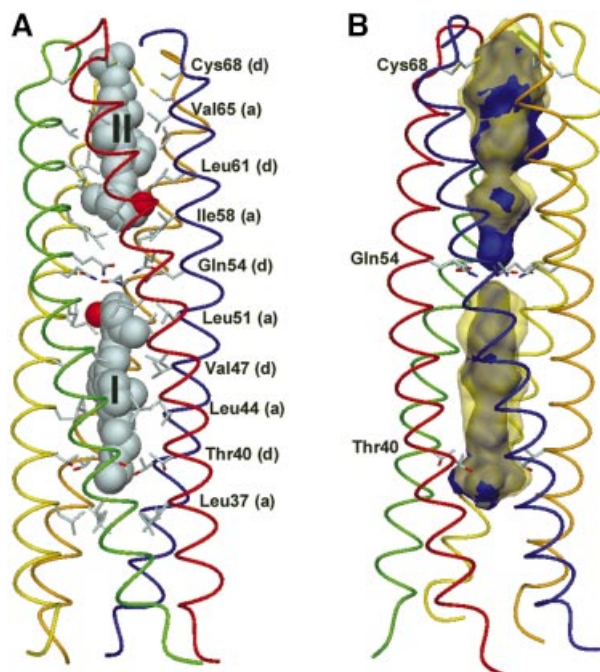


Fig. 1. Overall structure of COMPcc in complex with vitamin D₃. (A) Side view of the pentameric channel with bound ligand molecules I and II shown by van der Waals spheres. The N-terminus is at the bottom and the helical backbone is shown in ribbon representation with different colours for each chain. Amino acid residues forming knobs into holes according to the heptad repeat pattern are shown in atom type with a and d positions indicated in parentheses. The required volume occupied by each vitamin D₃ is 482 Å³. (B) Side view with helical backbone in ribbon representation and chains of emphasized amino acids shown in atom type. Two large cavities of the uncomplexed structure (blue) of volumes ranging from 450 Å³ (N-terminal) to 515 Å³ (C-terminal) can be seen. In the complexed structure, the volume inside the central channel is enlarged up so that the cavity volumes (yellow transparent) are increased markedly during diffusion of vitamin D₃ into the channels. The volumes are 667 Å³ (N-terminal) and 693 Å³ (C-terminal), respectively. The cavities have been determined using native COMPcc (1vdf) and COMPcc from the complex without vitamin D₃, performing MSMS with a sphere radius of 1.5 Å (Sanner *et al.*, 1996).

analysed by non-reducing SDS–PAGE, which, beside the fully oxidized pentamer band, also showed bands corresponding to the tetramer and the unclosed pentamer that runs slightly higher than the closed pentamer (data not shown) (Efimov *et al.*, 1996). We have observed incomplete oxidation of the bacterially expressed COMPcc frequently in our laboratory, indicating that *in vitro* reoxidation is a methodologically limiting step in the generation of a fully disulfide-linked COMPcc pentamer. Non-reducing SDS–PAGE of solubilized COMPcc–vitamin D₃ crystals revealed that the tetrameric and unclosed pentameric forms had been the preferred species during complex formation with vitamin D₃, reflecting the situation in the presented solution structure of the complex.

The crystal structure of the COMPcc–vitamin D₃ complex was solved at 1.7 Å resolution by molecular replacement using native COMPcc (Protein Data Bank code 1vdf) (Efimov *et al.*, 1996; Malashkevich *et al.*, 1996) as search template (Table I). The COMPcc chain fragment forms a parallel left-handed coiled-coil pentamer with an average length of 70 Å and an average outer

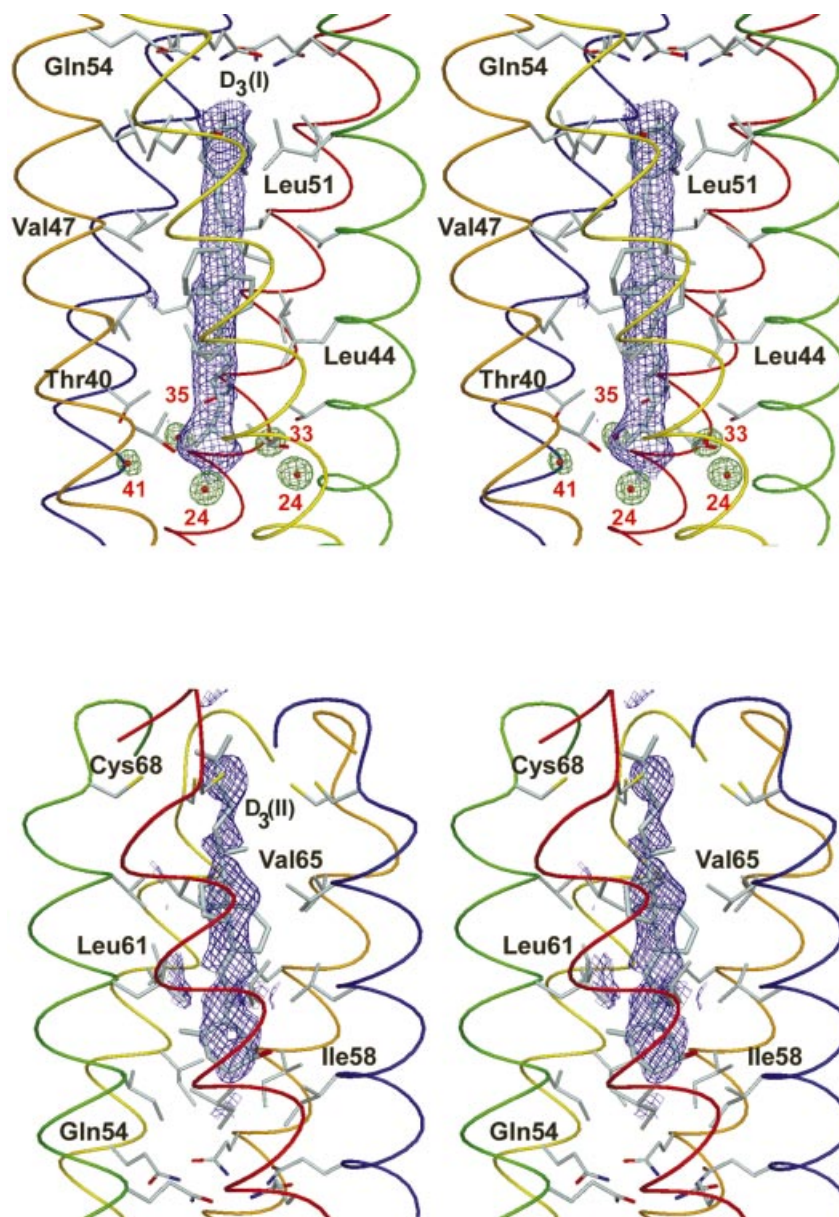


Fig. 2. Stereo view of the 1.7 Å resolution $2F_o - F_c$ omit map (1.2 σ contour level) superimposed on both vitamin D₃ molecules (molecule I at the top and II at the bottom). The five water molecules surrounding the dimethyl group of molecule I are shown as red balls and green electron density maps. The *B* values of the finally refined structure are shown in red.

diameter of ~30 Å. The axial pore of the pentamer is divided by the hydrophilic Gln54 ring system into two hydrophobic cavities exclusively lined with aliphatic side chains (Malashkevich *et al.*, 1996). In the COMPcc-vitamin D₃ complex structure, one molecule of vitamin D₃ is bound inside of each hydrophobic compartment in an elongated conformation, with the length axis of the ligands paralleling the channel symmetry (Figure 1A). Vitamin D₃ binding leads to an increase of both cavity volumes by ~30%, as demonstrated by the overlay of the areas in the occupied (yellow) and unoccupied (blue) (Malashkevich *et al.*, 1996) caverns in Figure 1B. The vitamin D₃ molecules are located on different sides of the Gln54 ring system in a head-to-head configuration with the 3-hydroxy groups of the A rings (steroid notation) pointing towards the central hydrophilic

narrowing. The N-terminal ligand molecule (I) is located between Thr40 and Leu51, and the second vitamin D₃ (II) is positioned between Ile58 and Cys68 at the C-terminus (Figure 1A). The electron density for the protein is well defined with exceptions in chain D showing disordering of the last seven C-terminal residues (66–72). Accordingly, the ring of interchain disulfide bridges between cysteines 71 and 68 of two neighbour chains is disrupted at this position leaving chain D disconnected in the otherwise fully oxidized pentamer. We believe that for the entrance of ligand II via the C-terminus, the state of interchain disulfide bridging is critical, considering the existence of only one ligand in the COMPcc-all-*trans* retinol complex located at the N-terminus (Guo *et al.*, 1998). A diffusion via the N-terminus can be excluded for molecule II because of the diameter limitation at the Gln54 ring system

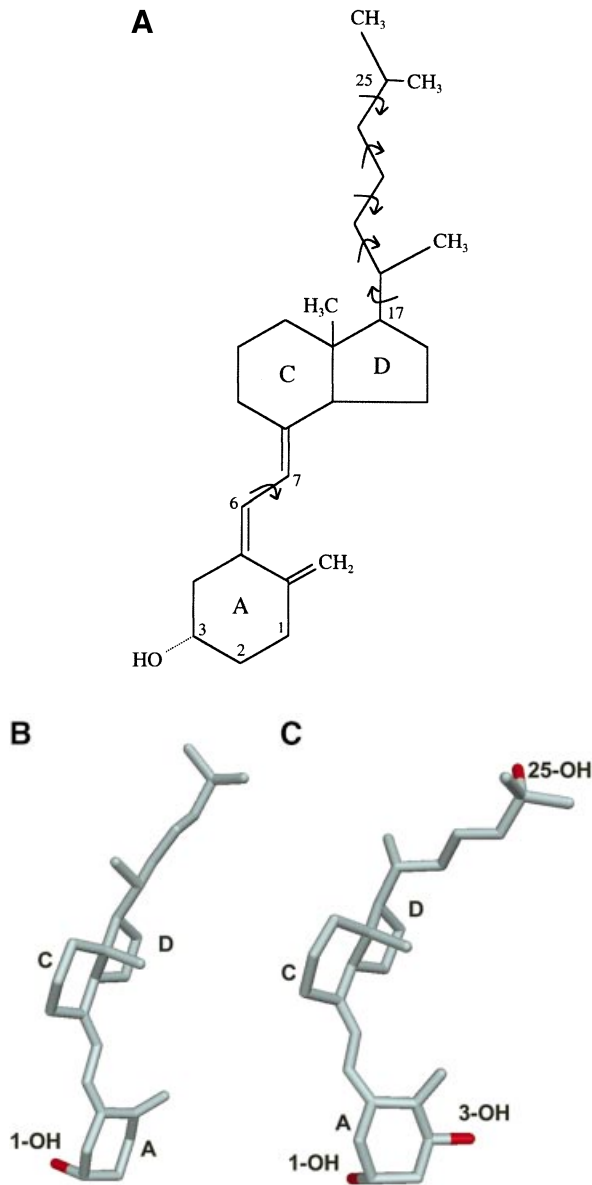


Fig. 3. Conformational flexibility of the vitamin D molecule. (A) Chemical formula of the vitamin D₃ molecule used for co-crystallization with COMPcc. The dynamic rotation around the single carbon-carbon bonds is indicated by curved arrows. The molecule is drawn in an elongated conformation, as in the COMPcc. (B) Atomic model of vitamin D₃ as a free molecule (Suwinska and Kutner, 1996). (C) Atomic model of 1 α ,25(OH)₂D₃ bound to the VDR LBD (Rochel *et al.*, 2000; PDB code 1DB1). The bound molecule shows a more bent overall shape with a dihedral angle of -149°. Ring systems and hydroxyl groups are labelled according to steroid notation.

with 2 Å. Likewise, a fully oxidized pentamer would not be accessible for a vitamin D₃ molecule via the C-terminus due to size restrictions. A diffusion via the N-terminus for ligand I, however, seems to be plausible. This assumption is underlined by changes within the helical backbone at the very N-terminus and re-orientations at the Thr40 ring to accommodate the dimethyl group fixation. These facts taken together suggest that the N-terminal position of ligand I is the primary binding site in the COMPcc for hydrophobic compounds, although data concerning the

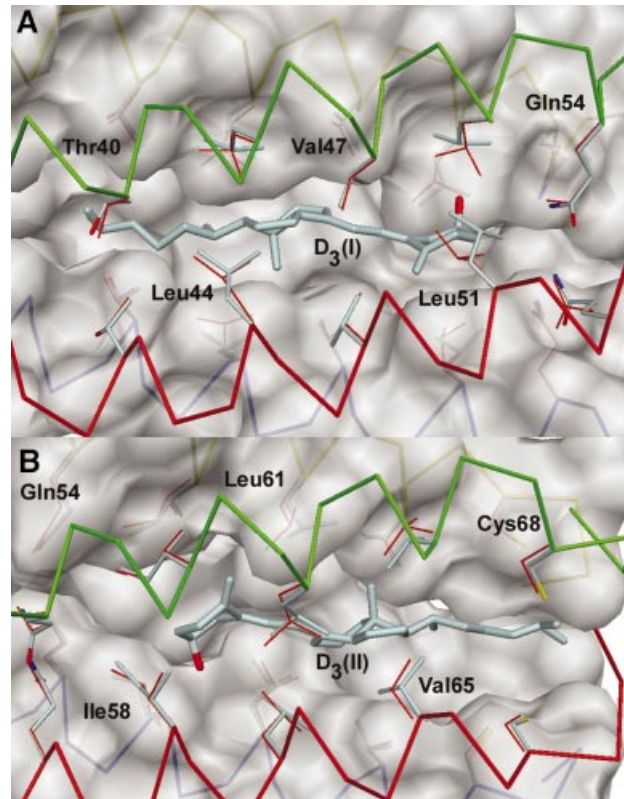


Fig. 4. Orientation of aliphatic side chains for both parts of the COMPcc channel in complex with vitamin D₃ molecules I and II in comparison with ligand-free COMPcc. Side views of the pentameric channel are shown with the N-termini on the left. In the protein part, three helices are shown by van der Waals spheres, and ribbon presentation is used for the main chain of the fourth (green) and fifth (red) helix. (A) Coordinates shown for ligand molecule I in the N-terminal channel. (B) Coordinates shown for ligand II at the C-terminus. Ligand molecules are highlighted and represent the predominant orientations resulting from an extensive refinement cycle (see Materials and methods). The side chains of the complexed structure are coloured according to atom type, native COMPcc (1vdf) side chains are shown in red. The most remarkable change occurs at Leu51, where all five side chains have to be spatially re-oriented to accommodate the A ring of vitamin D₃.

oxidative state of native COMP in cartilage are still lacking.

Conformation of the bound vitamin D₃ molecules and interactions with the COMPcc channel

The ligand molecules in the COMPcc-vitamin D₃ complex adopt a linear conformation with a length of ~17 Å separating the 3-OH group of the A ring (steroid notation) and the dimethyl group at carbon C25 (Figure 2). Their conformations resemble that of vitamin D₃ in the X-ray structure of the free molecule (Figure 3B) (Suwinska and Kutner, 1996). Both ligands are rather tightly bound, with the A rings adopting a chair B conformation and the 3-hydroxy groups in axial orientation (Bouillon *et al.*, 1995), directed from opposite sides towards the Gln54 ring system. The Cl⁻ ion described as being bound at this position in the uncomplexed COMPcc structure (Malashkevich *et al.*, 1996) is missing, although polar interactions of the 3-hydroxy groups with the proposed

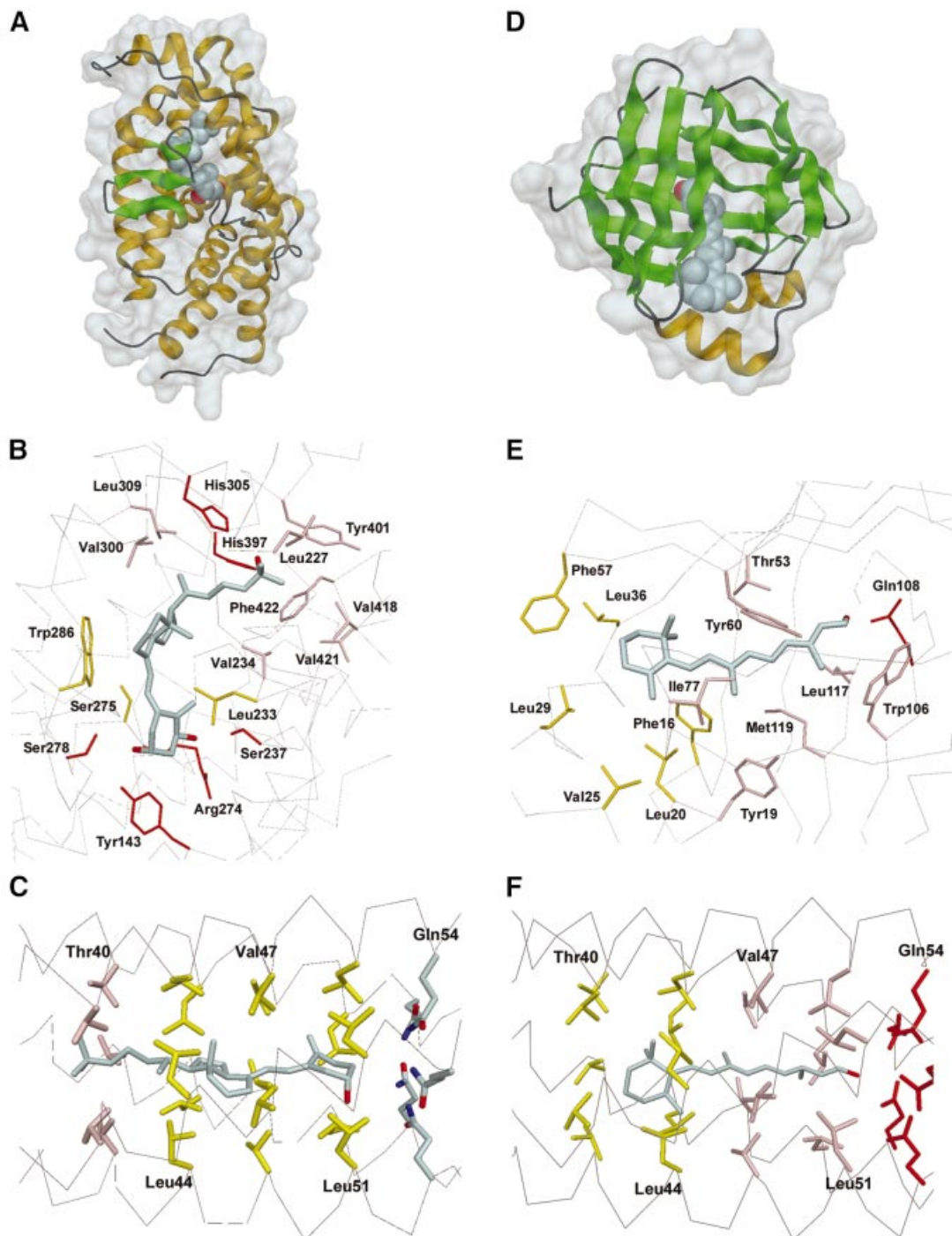


Fig. 5. Structural comparison of binding modes for vitamin D metabolites and all-*trans* retinol by their respective receptors and COMPcc. (A) Overall view of the VDR-1 α ,25(OH)₂D₃ complex (Rochel *et al.*, 2000). (B) Detailed view of the molecular binding mechanism of the VDR LBD. (C) Binding of vitamin D₃ by the N-terminal part of the COMPcc channel. (D) Overall view of the CRBP-all-*trans* retinol complex. (E) Detailed view of retinol binding in CRBP (Cowan *et al.*, 1993; PDB code 1CRB). (F) Vitamin A binding in COMPcc (Guo *et al.*, 1998; PDB code 1FBM). In the detailed views, residues involved in electrostatic interactions are in red, those in van der Waals distance to tail regions in pink, and residues in ring fixation in yellow. The ligand molecules are highlighted in render mode.

dipole system can be excluded due to disfavoured force field parameters.

The aliphatic side chains inside of the coiled coil cause 10 regular constrictions to the diameter of the pore, varying in size between 2 and 6 Å as defined by the van der Waals radii. The space-filling A and C-D ring systems (steroid notation) of the ligands fit into cavities flanked by

core residues in heptad repeat positions a and d, Leu51(a)/Val47(d) and Val47(d)/Leu44(a) for molecule I, and Ile58(a)/Leu61(d) and Leu61(d)/Val65(a) for molecule II. The opening of the unliganded channel had been described to be maximal at positions Val47 and Val65 (Malashkevich *et al.*, 1996), which here serve to accommodate the bulky C-D ring systems of the vitamin D₃

molecules. The seco B ring with the conjugated triene is tightly fitted into a hydrophobic ring of β -branched residues in heptad position d, Val47 for ligand I and Leu61 for ligand II. This interaction imposes a planar 6-*s-trans* conformation on the vitamin D₃ molecules in the COMPcc. The C6–C7 bond exhibits a torsion angle (C5–C6–C7–C8) of 178° that coincides with the α face of the A ring. The methyl C18 on the β face of the C ring points towards Leu44 (I) and Val65 (II) in close van der Waals contacts to the side chain atoms of three of the five chains (distances between are 3.3 and 3.6 Å). The 17 β aliphatic side chains are located in the hydrophobic cavities between Leu44/Thr40 (I) and Val65/Cys68 (II), and the remaining pocket of the channel is large enough to accommodate different variations of the long tail. The terminal dimethyl groups are fixed by residues Thr40 (d) for molecule I and Cys68 (d) for II.

The most remarkable change caused by the incorporation of the ligands is the increase in the cavity volumes within both channels. Analysis of the overall coiled-coil parameter for COMPcc revealed no significant change either in terms of the coiled-coil radius (8.57 Å for wild type in contrast to 8.59 Å for the complexed structure) or in terms of the supercoil pitch length of ~204 Å for both molecules. The atomic model, when compared with native COMPcc, shows r.m.s.ds on C α atoms of 0.69 Å. A further comparison revealed an increase of 0.2–0.3 Å in C α positions for Thr40–Leu51 and Ile58–Val65, respectively. This rather small enlargement for each individual amino acid adds synergistically to a cumulative effect. In addition, the adaptation of the protein core is realized more exclusively by a rotamer re-orientation of β -branched side chains forming knobs into holes (Figure 4). Basically, Leu44, Leu51 for molecule I and Ala65 for molecule II show different κ 1 and κ 2 angles of their rotamers.

Discussion

Biological implications

The steroid hormone 1 α ,25(OH)₂D₃ is the main stimulator of skeletal development and bone mineralization (Bouillon *et al.*, 1995; Malloy *et al.*, 1999), but it also induces cell differentiation and proliferation in various tissues (Walters, 1992). Potential therapeutic applications of 1 α ,25(OH)₂D₃ are treatment of renal osteodystrophy (Gal-Moscovici *et al.*, 2000), osteoporosis (Kleerekoper and Schein, 2001) and psoriasis (van De Kerkhof, 2001). 1 α ,25(OH)₂D₃ is also used in numerous cancer therapy models that include the systemic or oral administration of its activated form (Beer *et al.*, 2001; Hara *et al.*, 2001). The incidence of hypercalcaemia as a severe side-effect of such treatments initiated the search for analogues of calcitriol, which differ in their cellular responses. These efforts are often hampered by the poor uptake of the hydrophobic compounds.

In this study we report the structural features of a vitamin D₃ binding motif that is part of an extracellular matrix protein predominantly expressed in bone-morphogenetic tissue. The property of the COMPcc structure of adapting to different ligands, including vitamin D₃ and all-*trans* retinol, suggests a storage and delivery function of the COMP protein for signalling molecules relevant in cartilage tissue. Interestingly, it has been shown that the

accumulation of vitamin D₃ is highest in non-cellular components of bone, including extracellular matrix proteins (Neville and DeLuca, 1966). In a previous study, we demonstrated binding of vitamin D₃ to COMPcc by an increase in thermal stability of the coiled-coil pentamer (Guo *et al.*, 1998). The stabilizing effect of the hydrophobic compounds assayed in these experiments was more pronounced for larger molecules like vitamin D₃, with a change of the transition midpoint temperature of 8°C, than for smaller compounds such as benzene or cyclohexane ($\Delta T_M = 2^\circ\text{C}$). We concluded from this that binding of a prolate hydrophobic ligand inside the COMPcc channel should be obligatory because it is energetically favourable. Guo *et al.* (1998) reported an average binding constant of all-*trans* retinol to the COMPcc of ~6 μM . Regarding the higher T_M value of the COMPcc–vitamin D₃ complex, it can also be concluded that the binding affinity of vitamin D₃ to the N-terminal cavity of the COMPcc is even higher.

Both vitamin-D₃ and all-*trans* retinol are poorly soluble in aqueous media, and their delivery to target cells has been shown to be dependent on various transport proteins (Blomhoff *et al.*, 1990; Bouillon *et al.*, 1995). For the cellular uptake of vitamin D metabolites, different pathways are discussed (Willnow and Nykjaer, 2002). Besides free diffusion through the plasma membrane, recent studies have revealed carrier-dependent endocytic pathways playing a role in renal uptake of 25-hydroxyvitamin D₃ (Nykjaer *et al.*, 1999, 2001). A second type of interaction of vitamin D metabolites with the cell surface is defined by direct binding to membrane proteins leading to rapid activation of intracellular second messengers (Rosner *et al.*, 1999; Nemere *et al.*, 2000a). The expression pattern of COMP, which is restricted to growth regions of cartilage while being absent in resting and hypertrophic zones (Smith *et al.*, 1997), would point to a function of COMP in enhancing the availability of hydrophobic growth factors by increasing their local concentration in an avascular tissue. Farquharson *et al.* (1998) could demonstrate that the promotion of chondrocyte matrix production by ascorbic acid is connected with the synthesis of calcitriol and the upregulation of VDR expression. Also, some phenotypic features in the clinical manifestations of PSACH and MED, like growth retardation, resemble those in patients with hereditary 1 α ,25(OH)₂D₃-resistant rickets (HVDRR), a disease caused by defective intestinal calcium absorption as a result of mutations in the VDR gene (Malloy *et al.*, 1999). A lack of 1 α ,25(OH)₂D₃ function caused by mutated COMP in PSACH and MED might therefore serve as an explanation for this observation.

Comparison of COMPcc with other vitamin A and D binding proteins

Vitamin D and its numerous metabolites exhibit an unusual conformational flexibility (Figure 3A), which obviously gave rise to an array of differently shaped LBDs in receptor molecules and metabolizing enzymes (Norman *et al.*, 2001). Binding of 1 α ,25(OH)₂D₃ and all-*trans* retinoic acid to the nuclear vitamin D receptor and the retinoid X receptor (RXR) is defined both by electrostatic interactions via fixation of the respective hydroxy functions and hydrophobic contacts that stabilize the different

ring systems and aliphatic tail regions. All LBD–vitamin D X-ray structures known so far exhibit the *s-trans* conformation of the conjugated triene as a basic conformation. Deviations from this, which involve rotations around the C6–C7 bond and the five side chains, serve in adapting to a biologically active conformation as in the VDR/LBD. The ligands in the COMPcc–vitamin D₃ complex exhibit a conformation with a planar diene C5=C6–C7=C8 moiety (Figure 4), according to the lowest energy form displayed in the structure of free vitamin D₃ (Suwinska and Kutner, 1996) (Figure 3B). This is in contrast to the curved shape ('twisted bowl') of 1 α ,25(OH)₂D₃ in the VDR (Rochel *et al.*, 2000; Tocchini-Valentini *et al.*, 2001). The more bent geometry of 1 α ,25(OH)₂D₃ in VDR (C6–C7 torsion angle of –149°) would cause steric clashes inside the tight COMPcc channel (Figure 3C).

Remarkably, the electron density for both ligand molecules in the COMPcc–vitamin D₃ complex is less resolved than for the protein part of the structure (Figure 2). The C–D ring systems are somewhat diffused, suggesting that orientation is not unique. Despite this disordering of vitamin D₃, only one predominant binding conformer exists in both cases. The appearance of five water molecules between Leu37 and Thr40 showing slightly different *B* values and shape of the electron density map suggests an incompatibility with the 5-fold symmetry of COMPcc. This only partial breakdown of local symmetry can explain the ambiguous orientation of vitamin D₃ towards COMPcc. As COMPcc is proposed as a storage system for vitamin D, there is no need to fix the ligand molecule in a predominant orientation.

The overall structure of the VDR LBD matches the pattern of other nuclear receptor LBDs with specific deviations due to the widening of the ligand binding pocket (Rochel *et al.*, 2000). The ligand is tightly wrapped by 13 α -helices arranged in three layers and a three-stranded β -sheet (Figure 5A). Each of the three hydroxyl groups forms two hydrogen bonds: 1-OH with Ser237 and Arg274, 3-OH with Tyr143 and Ser278, and the 25-OH with His305 and His397 (Figure 5B). The conjugated triene, connecting the A and C rings, is fitted in a hydrophobic channel sandwiched between Ser275 and Trp286 on one side and Leu233 on the other side. Specific interactions involve the hydrophobic contacts of the 17 β -aliphatic chains with a cluster around Val418 on one side and Val300 on the opposite side.

In serum, vitamin D₃ and its various metabolites, including 1 α ,25(OH)₂D₃, are delivered in a complex with vitamin D binding protein (DBP). The solution structure of this carrier protein revealed a partly solvent-exposed cleft functioning as the vitamin D binding site (Verboven *et al.*, 2002), which is in contrast to the closed binding pocket provided by the VDR and the COMPcc presented in this study. This circumstance might also reflect the moderate binding affinity of DBP for 25-hydroxyvitamin D₃ ($K_a = 10^7$ – 10^9 M⁻¹) (Head *et al.*, 2002).

CRBP reveals a 10-stranded antiparallel β -barrel that encapsulates the ligand inside of a lipophilic cavity (Cowan *et al.*, 1993). All-*trans* retinol is bound along the barrel axis with the hydroxyl group lying in the centre of the barrel (Figure 5D). The retinol molecule is almost planar with the β -ionone ring double bond in *trans* position

to the isoprene tail. The hydroxyl group is positioned within hydrogen-bonding distance of the Gln108 side chain (Figure 5E). The β -ionone ring fits into a hydrophobic niche formed in particular by the side chains of Phe16, L20, Val25, Leu29, Leu36 and Phe57. The isoprene tail is flanked by residues around Tyr119 and Tyr60, and fixed by Leu117 together with Trp106.

A critical feature is the hydrogen bonding of terminal hydroxy functions in both structures. Both the three frontal (1-OH, 3-OH and 25-OH) OH groups in 1 α ,25(OH)₂D₃ and the terminal OH group in retinoic acid are fixed via electrostatic interactions. In COMPcc, the Gln54 ring system can be discussed to determine the orientation of the ligands, but it does not add significantly to binding. Rather, the fixation of both ligand molecules is enforced by the spatial arrangement of the cavities between core layers in a and d positions (Figure 5C). We have demonstrated earlier complex formation of COMP with all-*trans* retinol by the corresponding X-ray crystal structure, which showed a positioning of the β -ionone ring in a hydrophobic environment near Thr40 at the N-terminus and a fixation of the terminal hydroxyl group within the Gln54 ion trap (Figure 5F) (Guo *et al.*, 1998). A mutant COMPcc in which Gln54 is replaced by Ile was found to bind all-*trans* retinol with similar affinity as the wild-type molecule (Guo *et al.*, 1998). We conclude from this that the alternating pattern of hydrophobic core residues is the driving force for the fixation of both ligand molecules within the channel. The position of ligands in both binding compartments is therefore adjusted by core residues in heptad positions a and d.

Conclusion and perspective

In the present study as well as in earlier works, by the presentation of binding data and X-ray solution structures of the respective complexes, we have demonstrated the association of hydrophobic signalling molecules with the recombinant coiled-coil domain of COMP. In regard to the importance that the assayed morphogens have in limb formation, the possibility of an extracellular matrix protein to mediate their cellular delivery is an attractive model. To assess this proposed functional property of COMP in cartilage, we intend to extract and purify the protein from bovine articular cartilage in native condition. The separation of the lipid phase from this COMP sample and its analysis by mass spectroscopy should help to reveal the physiological role of COMP as suggested by our data.

Materials and methods

Expression and purification of recombinant COMPcc

The coiled-coil domain of rat COMP comprising residues 27–72 was prepared as described previously (Efimov *et al.*, 1996; Guo *et al.*, 1998). Formation of the pentameric complex after purification was facilitated by disulfide reshuffling using a glutathione redox system and checked by non-reducing SDS–PAGE. Oxidized COMPcc was dialysed against PBS pH 7.4 and adjusted to 1 mg/ml. Solid vitamin D₃, obtained from Fluka, was added to 1 mg/ml protein solution and the mixture was shaken for 24 h at 4°C in the dark to facilitate equilibration of binding. Unbound vitamin D₃ was removed by ultracentrifugation and the solution was concentrated to 10 mg/ml COMPcc with a Vivaspin (Vivascience) device.

Crystallization

Crystallization experiments were performed at room temperature employing the vapour diffusion technique. Hanging droplets were made by mixing 2 µl of protein solution (10 mg/ml) with 0.2 M sodium acetate, 0.1 M Tris-HCl pH 8.5 and 30% PEG 4000. The crystals belong to space group $P2_1$ and contain one molecule of the pentameric COMPcc within the asymmetric unit.

Data collection and processing

The high resolution data set was collected at synchrotron DESY (BW7B) on a MAR research imaging plate detector. Diffraction images were processed using program suite MOSFLM (Leslie, 1994) and the structure factors were scaled and reduced using SCALA from the CCP4 package (CCP4, 1994). Statistics of the merged data are given.

Structure determination and refinement

Molecular replacement was performed using the AMoRE program of the CCP4 package (CCP4, 1994). A poly-serine model of native COMPcc structure (Ivdf) was used as search template. Positional refinement was performed with CNS using the maximum likelihood method (Brunger *et al.*, 1998). Ten percent of the reflections were excluded for use in a cross-validation set. Refinement with CNS was alternated with manual electron density refitting of side chains and terminal regions using MAIN (Turk, 1992). After including all native amino acid residues, the R_{free}/R value fell to 32.8/29.7. At this stage, the ligand molecules have been fitted into a 3.0σ contoured $F_o - F_c$ difference map using the coordinates of the free vitamin D₃ molecule (Suwinska and Kutner, 1996). To determine the favoured axial orientation of the ligands within the pentameric channel, a 2° stepwise refinement (conjugated gradient minimization together with individual B-factor refinement) along the 5-fold local symmetry axis was performed. Interpretation of the electron density maps for each solution together with monitoring of the R_{free}/R value ratio revealed that one orientation is preferred, which is shown in Figures 2–5. In further refinement, overall anisotropic B-factor and bulk solvent corrections were utilized. Simulated annealing omit maps confirmed the correctness of the protein and ligand structures. At R values of 27.1/25.0, water molecules were added, chosen by distance criteria and hydrogen bonding geometry, and were tested for position in spherical density, reasonable temperature factors, real-space R values and improvement of the R-factors. The final structure contains 245 water molecules. The final R-factor and R_{free} -factor are rather high (25.4 and 23.1%, respectively), which can be explained by the fact that the C-terminal part of chain D was found to be disordered and that both ligand molecules could be refined only in one predominant orientation within the channel.

The co-ordinates for the structure have been deposited in the Protein Data Bank under accession code 1MZ9.

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