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Deciphering protein dynamics changes along the pathway of retinol uptake by cellular retinol-binding proteins 1 and 2

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ABSTRACT

Four Cellular Retinol-binding Proteins (CRBP 1, 2, 3, 4) are encoded in the human genome. CRBP 1 and 2, sharing a 56% amino acid sequence identity, exhibit the highest binding affinities for retinol. Previous NMR studies provided some insights into the mechanism of retinol uptake, but details of such mechanism remain to be elucidated. Herein, the results of molecular dynamics simulations for the uptake of retinol by CRBP 1 and 2 are consistent with the presence of two different retinol entry points, both involving the 'cap region' (α-helices I and II and neighboring loops). We observed that a hydrophobic patch at the surface of the 'portal region' (α-helix II, CD and EF loops) of CRBP 1 attracts retinol, which accesses the binding cavity through an opening generated by the concerted movements of Arg58 and Phe57, present in the CD loop. In CRBP 2 a different distribution of the surface residues of the 'cap region' allows retinol to access the binding cavity by sinking in a hydrophobic matrix between the two α-helices. Polar interactions mainly affect retinol movements inside the β-barrel cavities of both CRBPs. The interaction energy profiles are in agreement with the different behavior of the two protein systems.

1. Introduction

The transport of the very insoluble and chemically unstable retinol molecule in the physiological environment requires the presence of specific intracellular carrier proteins (the Cellular Retinol-binding Proteins, CRBPs) to solubilize and protect the vitamin [1,2]. CRBPs belong to the superfamily of intracellular Lipid-binding Proteins (iLBPs) [3,4], whose different members, despite their rather low amino acid sequence identities, possess a highly conserved three-dimensional structure, while exhibiting high ligand binding specificities [5]. iLBPs are single-domain proteins, whose structure mainly consists of an antiparallel β-barrel, which accommodates the hydrophobic ligand, and two α-helices, which close up one end of the binding cavity and form a 'cap' with the neighboring loops. Two regions may represent possible ways of uptake of the ligand in iLBPs: one is the so called 'portal region', which is formed by helix II (residues 27–35, according to human CRBP 1 and 2 residue numbering) and CD and EF loops (residues 55–58 and 75–79, respectively) and is the most flexible region at the protein surface, as suggested by NMR and X-ray diffraction studies [6–9]; the other one is the so called 'gap region', formed by D and E β-strands (residues 59–65 and 68–74, respectively), which are not hydrogen-bonded to each other, so that the interruption of the β-barrel thus generated might provide a point of flexibility in the structure, suitable for the uptake of the ligand [10,11].

Four CRBPs (CRBP 1, 2, 3 and 4), encoded in the human genome, are characterized by different tissue distributions and remarkably different affinities for retinol [12–16]. In particular, CRBP 1 is widely expressed in several tissues in which it functions as regulator of retinol homeostasis [15,17], while the expression of CRBP 2 is limited to the small intestine, where this isoform is involved in the intestinal regulation of vitamin A absorption and metabolism [14]. An apparent Kd value of approximately 2 nM was obtained by two different groups for CRBP 1 by means of fluorometric titration [18,19]. However, it has been estimated that the Kd value for the interaction of retinol with CRBP 1 can be at least one order of magnitude lower, as determined by the competition between retinol and retinal for the binding to CRBP 1 [18]. With regard to the difference in retinol binding affinity between CRBP 1 and CRBP 2, evidence has been obtained of similar binding affinities of the two proteins, as determined by means of fluorometric titrations ([14], and references therein). However, a direct competition of CRBP 1 and CRBP 2 for retinol suggested that the affinity of CRBP 1 may be 100-fold greater than that of CRBP 2 [20]. The interactions of retinol with CRBP 1 and 2 have been widely characterized structurally [9,21–24], but the reason for the different binding affinity of retinol has

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Abbreviations

ALBP	Adipocyte Lipid-binding Protein
CRBP	Cellular Retinol-binding Protein
FABP	Fatty Acid-binding Protein
I-FABP	intestinal Fatty Acid-binding Protein
iLBP	intracellular Lipid-binding Protein
MD	molecular dynamics
NMR	nuclear magnetic resonance
RMSD	root mean square deviation
RMSF	root mean square fluctuation

not yet been clarified. In CRBP 1, and possibly CRBP 2, the importance of the two conserved key binding residues Lys40 and Gln108 is demonstrated by the fact that their replacement leads to a substantial loss of binding affinity for retinol [9].

In a previous work [25], the involvement of the amino-aromatic interaction between Gln108 and the close residue Phe4 in CRBP 1 was tested. Since Phe4 is replaced by a Gln in CRBP 2, this substitution was considered to be crucial to explain the different affinity of the two isoforms. Unexpectedly, for the Q4F/CRBP1 and F4Q/CRBP2 mutant forms the same Kd possessed by their respective WT forms were determined. It still remains to be clarified which factors are responsible for the different affinity of CRBP 1 and 2 for retinol.

The mechanism of ligand uptake in iLBPs has been a broadly studied theme, but to date only hypotheses have been made [26] regarding this issue. X-ray studies on several iLBPs, including CRBP 1 and 2 ([9,24], and references therein), have shown that the cavity of these proteins is inaccessible, so that protein conformational changes are needed for ligand uptake ([26], and references therein). Several NMR studies were performed on different iLBP members [27–29,7,30], but only in few cases such studies provided indications on the pathway of ligand uptake. In particular, NMR studies on rat CRBP 1 and 2 indicated the high flexibility of the 'portal region', thereby suggesting its involvement in the mechanism of ligand uptake [31]. In particular, a different flexibility of the helix-turn-helix motif, which in CRBP 1 seems to be restricted to the helix II, while in CRBP 2 also concerns helix I, was observed [7]. However, the uptake mechanism remains unclear and only for CRBP 1 a model was proposed, according to which small conformational changes, involving Arg58 and Phe57, may be sufficient for the entry of retinol [31].

Several molecular dynamics simulation studies were carried out to investigate the protein dynamics of iLBPs [11,32–34] [33] with the ultimate purpose of clarifying the uptake mechanism of ligands. However only few MD investigations were carried out on CRBPs [10,35] and none concerned the uptake of a ligand. Herein, we report on MD simulations of the apo forms of the two primary cellular retinol-binding proteins isoforms, CRBP 1 and 2, to study their structural flexibility and, in particular, to identify specific protein regions possessing the highest degree of this flexibility, suggesting their involvement in retinol uptake. We have then simulated the dynamics of CRBP 1 and 2 in the presence of retinol, exploring the pathways of access of the vitamin to its β-barrel binding cavity.

2. Computational details

2.1. Molecular dynamics simulations

All MD simulations were performed by means of GROMACS 4.5.5 software [36,37], using the force field Gromos 53A6 [38]. Starting coordinates for apo-CRBPs were obtained from the Protein Data Bank (PDB ID: 5LJK and 2RCQ for human apo-CRBP 1 and 2, respectively). For each structure, water and all the other molecules present in PDB files were manually removed. For the simulations in the presence of retinol, we used the same apo-protein starting structures, to which one retinol molecule was added by means of the VMD graphical software [39]. For retinol topology, the parameters already present in the G53A6 force field were used. Since cellular retinol binding proteins are cytoplasmic proteins, we performed all simulations embedding proteins in a water box, keeping a 1 nm thick water layer around the macromolecule and adding enough Na^+ and Cl^- ions to neutralize the system and to reach a physiological 0.1 M concentration for both ions. During the simulations, the periodic boundary conditions were applied to the system. The Berendsen barostat was used for pressure coupling [40] and the V-rescale thermostat for temperature coupling [41]. For each system, internal constraints were relaxed by an energy minimization of the whole system. Then, a position restrained MD simulation 50 ps long, was performed to relax solvent molecules around the protein.

2.2. Simulations in the absence of retinol

For the apo-protein simulations, aimed at investigating overall and local protein structural flexibilities, full 100 ns MD runs were carried out at 300 K and 1 atm, with an integration step of 2 fs.

2.3. Simulations in the presence of retinol

Several preliminary simulations were performed to better define optimal conditions suitable for monitoring retinol uptake and to explore the role of hydrophobic patches around the "cap region", changing every time the initial position of retinol. Due to its lipophilicity, retinol was found to initially interact with hydrophobic regions at the protein surface. In fact, in CRBPs many hydrophobic patches are present on their surface so that the lipophilic vitamin remained trapped for a long time bound to such patches during a simulation. For this reason, we increased the temperature to supply energy to the system, thus overcoming possible potential barriers, consistent with the method of temperature-accelerated molecular dynamics [42,43]. Therefore, we carried out three MD simulations on human CRBP 1 and 2 at 350 K and 1 atm, in the presence of one retinol molecule, changing every time the initial position and orientation of retinol near the "cap region" (see Figs. S4 and S5). In each attempt, the ligand was able to access the binding pocket through the same entry point (different in CRBP 1 and 2). The uptake of the ligand was found to take place in less than 40 ns for both CRBPs, even if in two cases the simulation time was increased to 60 ns, to allow the ligand to be deeply inserted (see also the Supplementary Material).

2.4. Binding energy analysis

In the simulations in the presence of retinol, the analysis of the different components of the energy involved in the association of retinol to the protein was performed by means of the MM/PBSA method implemented for GROMACS, available in the g_mmpbsa software package [44] (http://rashmikumari.github.io/g_mmpbsa/), coupled with the APBS package [45]. The tool calculates the components of the binding energy (vacuum potential energy, polar solvation energy, non-polar solvation energy) in the single trajectory approach, with the exception of the entropic term, and for this reason it is unable to give the absolute binding energy. However, the tool is suited for calculating relative binding energies, for example to compare a ligand binding to similar receptor proteins [46], as in our case. In such condition, the difference in conformational entropy between the two complexes should be small. To calculate the average value of the binding energy (ΔG_{bind}), we used the equilibrated portion of the trajectory, in which both ligand position (inside the binding site) and protein conformation