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# Retinol Modulates Site-Specific Mobility of Apo-Cellular Retinol-Binding Protein to Promote Ligand Binding

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***Abstract:*** A fundamental question in protein science is how the inherent dynamics of a protein influence its function. If this function involves interactions with a ligand, the protein−ligand encounter has the potential to modulate the protein dynamics. This study reveals how site-specific mobility can be modulated by the ligand to facilitate high affinity binding. We have investigated the mechanism of retinol uptake by the cellular retinol-binding protein type I (CRBP) using line shape analysis of NMR signals. The highly similar structures of apo- and holo-CRBP exhibit closed conformations that seemingly offer no access to ligand, yet the protein binds retinol rapidly and with high affinity. NMR line shape analysis reveals how protein dynamics resolve this apparent paradox. An initial nonspecific encounter with the ligand induces the formation of long-lived conformers in the portal region of CRBP suggesting a mechanism how retinol accesses the cavity.

## 1. Introduction

Although protein−ligand interactions have been at the core of biochemical and biophysical research for decades, little is known about their fundamental mechanisms and the correlation between the internal dynamics and functions of proteins. New experimental evidence, provided by advances in nuclear magnetic resonance (NMR) spectroscopy, allows a reliable and sensitive investigation of ligand binding mechanisms on microsecond to millisecond ($\mu$s−ms) time scales using line shape analysis[1,2] and relaxation dispersion experiments.[3,4]

Recent NMR studies have tackled allosteric behavior,[5] enzymatic turnover,[6] and protein and ligand binding.[7−11] Despite detailed studies of protein dynamics, little is known about the mechanisms of how the internal motions of a protein can modulate its interaction with the ligand, especially when internal motions are on a different time scale compared to the kinetics of ligand binding.[12] A further debate concerns the kinetic mechanism of protein ligand interactions. Although folding intermediates could be detected for many proteins (see ref 13 and references therein), two-state models have been assumed in most analyses of protein interactions. Recent work has provided evidence for more complex mechanisms, involving sequential binding steps and the generation of slowly exchanging conformers by ligand-induced dynamics.[1,14] In one case, long-lived binding intermediates were directly observed in NMR line shapes.[11]

Cellular retinol-binding protein type I (CRBP), the most abundant and ubiquitous carrier of vitamin A in mammalian tissues, is a member of the intracellular lipid-binding protein (iLBP) family.[15−19] The solution structures of both the ligand-

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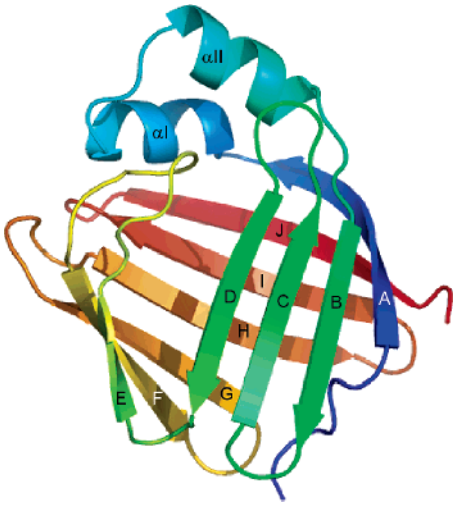


***Figure 1.*** Ribbon diagram of CRBP type I (19) depicting the 10-stranded $\beta$-barrel (A−J) and the two flanking α-helices, a typical fold for the iLBP family.

free (apo) and retinol-bound (holo) CRBP have been determined using high-resolution NMR spectroscopy[20,21] and found to be highly similar to the crystalline holo-form.[22] The typical overall structural topology of iLBPs, a $\beta$-barrel formed by 10 antiparallel $\beta$-strands (A−J) and two short α-helices which are inserted between $\beta$A and $\beta$B (Figure 1), encloses a binding cavity which is lined by polar and hydrophobic residues and contains a few ordered water molecules. In both apo- and holo-CRBP, the cavity within which all-trans retinol binds with high affinity is fully shielded from the outside medium.[20] This feature raises the question of how the ligand enters and is then sequestered by the carrier and how, after intracellular transport, it is released to the membrane-bound enzymes that catalyze its conversion to retinaldehyde,[23,24] the precursor of retinoic acid, the most active form of vitamin A.[25,26] Structures of other members of the iLBP family[27,28] have allowed speculation about a possible "portal" located between α-helix II and the two turns $\beta$C-$\beta$D and $\beta$E-$\beta$F, although no structure shows an open form of the putative portal. The higher picosecond to nanoseconed (ps−ns) mobility of some residues clustered in the portal region of apo-intestinal fatty acid-binding protein (I−FABP) with respect to the holo-form suggested a dynamic role of the portal region in regulating ligand entry.[29] However, this was not observed for CRBP.[20] Recent studies revealed more significant dynamic effects on a $\mu$s−ms time scale for apo-CRBP with particularly strong exchange effects in the $\beta$C-$\beta$D turn and in α-helix II.[21,30]

Although most exchange phenomena were in the fast exchange regime on the NMR time scale, the rates of exchange varied between 100 and 1000 $s^{-1}$. The high degree of conformational flexibility observed in the apo-protein was almost completely quenched upon binding of retinol. This observation gave rise to the question of whether the observed ms dynamics in the apo-form are essential for ligand uptake and how they can lead to conformations with access to the cavity.

In this study, we have used NMR line shape analysis to shed new light on the role of protein dynamics for retinol binding by CRBP, to discern different steps in a complex kinetic mechanism, and to further investigate the role of dynamics of residues in the putative portal.

## 2. Experimental Procedures

**Protein Expression and Purification.** CRBP was expressed and purified as described previously.[20] For NMR measurements, samples of 0.7 mM $^{15}$N-labeled CRBP were prepared in potassium phosphate buffer (20 mM, pH 6.0, 90% H2O/10% D2O) in a volume of 500 $\mu$L.

**Protein Titrations and Line Shape Analysis.** Retinol was solubilized in deuterated methanol ($CD_3OD$) and added to the $^{15}$N-labeled CRBP sample in small steps (typically 5 $\mu$L each) yielding mole fractions of the ligand with the following values: 0.02, 0.04, 0.15, 0.30, 0.50, 0.70, 0.90, and 1.10. A last addition of 1 aliquot of retinol solution confirmed the end of the titration. After each addition, a high-resolution sensitivity enhanced $^1$H/$^{15}$N−HSQC spectrum was recorded with 2048 × 700 data points at 11.7 T and a temperature of 298 K. Spectra were processed with zero-filling and 90° shifted cubic sine window functions. For residues which were not subject to chemical shift degeneracy, cross-sections in both spectral dimensions were extracted from all spectra by carefully selecting slices containing the signal maxima. Line shapes for both dimensions were simulated simultaneously for selected kinetic models using the set of eight experimental spectra as described in detail elsewhere.[2,11] NMRKIN was used to calculate the time domain signal for sections of HSQC spectra for a given kinetic mechanism assuming steady-state line shapes as described by the equations of Gutowsky and McConnell.[31,32] Chemical shifts of signals, line widths, populations, and kinetic rates were adjusted iteratively to achieve consistency with experimental data. In the slow exchange regime, only an upper limit of the off-rate can be assigned because the line shapes depend only slightly on the rate. Residues were assigned to the model that best described their line shapes.

## 3. Results and Discussion

In this study, we have analyzed NMR line shape changes caused by the interaction of CRBP with retinol to identify motions in the protein which are relevant for ligand uptake. Line shapes of resonances in $^1$H/$^{15}$N−HSQC spectra of CRBP which experience a chemical shift change for increasing concentrations of retinol represent a steady-state of the exchange processes between states of the protein involved in the interaction. Although a simple two-state exchange between free and bound protein will lead to line shapes similar to those shown in Figure 2a,b, it has been shown for other proteins that more complex mechanisms can easily be distinguished.[10,11,14] Quan-

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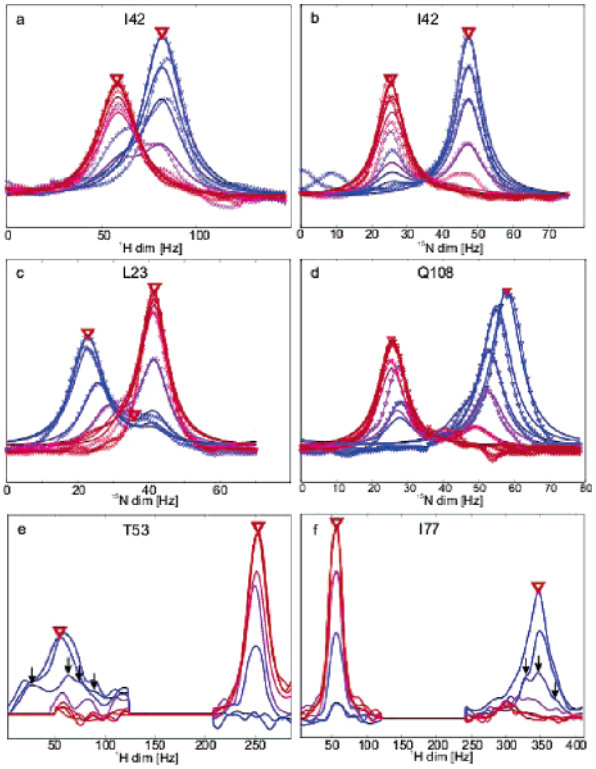


***Figure 2.*** $^1$H and $^{15}$N cross-sections of signals of CRBP in the titration with retinol. Experimental data marked with "▽" are depicted from blue to red for successive addition of the ligand. Simulated spectra are shown with solid lines. Resonance frequencies of the apo-protein (P), the holo-form (PL) and intermediates are marked by a red "▽". (a) $^1$H and (b) $^{15}$N line shapes of I42 were simulated according to simple one-step binding (eq 1). $^{15}$N cross-sections of (c) L23 and (d) Q108 require the more complex mechanism 2. For residues T53 (e) and I77 (f), experimental signals of apo-CRBP show complex line shapes in intermediate steps of the titration with retinol. Signal shoulders in the third spectrum are marked with arrows.

titative analysis yields kinetic parameters and a validation of the kinetic model selected for the simulation.

For 31 residues (see Table 1 and Figure 1 in the Supporting Information), we observed typical slow exchange line shapes that could be simulated employing a two-state binding mechanism

$$\text{apo-CRBP} \underset{\text{low } k_{off}}{\overset{\text{[retinol]}, k_{on}}{\rightleftharpoons}} \text{holo-CRBP} \qquad (1)$$

with second-order binding and first-order release ($k_{on}$ and $k_{off}$ are forward and reverse reaction rate constants). Panels a and b of Figure 2 show as an example for typical signal cross-sections in the $^1$H and $^{15}$N dimension of residue I42 located in $\beta$B of CRBP. In the first spectrum (blue), only one signal at the resonance frequency of apo-CRBP was observed. For successive addition of retinol, the intensity of the signal of apo-CRBP decreased, whereas a signal at the resonance frequency of holo-CRBP appeared and grew as the population of the complex increased. For residue I42, the upper limit for the off-rate is 10 $s^{-1}$, whereas the on-rate is outside the kinetic window accessible by this analysis.

These two-state line shapes do not answer the question how binding occurs, especially as the apo-form has no opening to allow ligand entry. Closer inspection of the line shapes provided an important clue. Astonishingly, line shapes of as many as 48 residues (see Table 1 and Figure 1 in the Supporting Information) showed additional complexity. As an example, line shapes obtained for L23, a residue in the linker region between the two helices, and for Q108, a residue in $\beta$H, are depicted in Figure 2, panels c and d, respectively. In both cases, the resonance frequency of the signal corresponding to apo-CRBP (blue) changed in successive titration steps indicating a fast exchange process ($k_{off} > 2000\ s^{-1}$). In contrast, signals of holo-CRBP (red) did not experience any ligand dependent chemical shift perturbation but only altered intensity, typical of slow exchange phenomena. This behavior can be explained by a two-step binding mechanism

$$\text{apo-CRBP} \underset{\text{high } k_{off}}{\overset{\text{[retinol]}, k_{on}}{\rightleftharpoons}} \text{CRBP*} + \text{retinol} \underset{\text{low } k_{off}}{\rightleftharpoons} \text{holo-CRBP} \qquad (2)$$

In this model, a fast exchange process involving the intermediate CRBP* with a high off-rate ($k_{off} > 2000 s^{-1}$) is sequentially followed by a slow step with a rate $<10\ s^{-1}$. The first step, initiated by the addition of ligand, may represent a low affinity interaction with retinol involving one or more molecules of the ligand followed by uptake of one retinol molecule into the cavity with high affinity. Considering the very different rates for the two steps of this reaction, the second step must be responsible for the high affinity of CRBP for retinol ($K_D \sim 0.1$ nM).

Even more interestingly, another type of complexity is observed in the line shapes of some residues in and around the putative portal region (see Table 1 and Figure 1 in the Supporting Information): their signals in intermediate steps of the titration exhibit several shoulders indicative of slow conformational exchange. Line shapes of T53 in the end of $\beta$C and I77 in turn $\beta$E-$\beta$F are shown in Figure 2, panels e and f, as examples (shoulders are indicated by arrows). This behavior is best explained[1,2] by a parallel scheme of the type

$$\text{apo-CRBP} \underset{\text{[retinol]}}{\overset{\text{[retinol]}}{\rightleftharpoons}} \begin{matrix} \text{CRBP}_1\text{*} + \text{retinol} \\ \text{CRBP}_2\text{*} + \text{retinol} \\ \text{CRBP}_3\text{*} + \text{retinol} \end{matrix} \rightleftharpoons \text{holo-CRBP} \qquad (3)$$

In this mechanism, $CRBP_i$* represents a series of protein conformers with small chemical shift deviations from the starting signal of the apo form. Therefore, this mechanism explains the complexity of signals after initial addition of ligand and the lack of complexity on the side of holo-CRBP: the final product is conformationally stable; therefore, the different conformers $CRBP*_i$ are all individually in slow exchange with a single holo-CRBP species although not all conformers may lead to the formation of holo-CRBP. From the close proximity of the signal shoulders, an upper limit for the exchange frequency between the different conformers can be estimated. Individual signal components for lines such as those shown in Figure 2, panels e and f, for T53 and I77 exhibit chemical shift differences between 7 and 25 Hz. The exchange frequencies are therefore less than 15−55 $s^{-1}$, depending on the residue. Interestingly, relaxation dispersion experiments of apo-CRBP showed a significant exchange contribution on the ms time scale for those residues.[21,30] For example, I77 showed conformational exchange with a rate of 390 $s^{-1}$. This observation suggests that ligand binding causes a transformation of chemical exchange from a millisecond time scale in the apo-protein to slow conformational exchange on a sub-second time scale in intermediate forms

***Table 1.*** Grouping of Residues According to the Binding Mechanism They Sense as Suggested by Their Line Shapes Simulation

| kinetic model | CRBP residues |
|---|---|
| slow exchange, two-state model (1) | 2,4,6,10,13,18−20,22,24,26,31,36,41,42,49,50,55,62,66−69,87,94,96,97,103,104,119,131 |
| two-step/three-state binding mechanism (2) | 3,5,7,8,15−17,23,25,27,29,30,32,35,39,40,53,54,59,63,64,74,78,82,83,85,93,98,100,102 105−109,111,113,115−118,120,126,127,129,130,132,134) |
| parallel binding mechanism (3) | 9,29,32,34,37,39,40,52,53,56−60,77,78,89,91 |

$CRBP_i$*. In other words, the encounter with the ligand is "freezing" the motion of residues located in the putative portal region of the protein. Residues exhibiting these types of complex line shapes extend into $\beta$-sheets A, B, C, and D. D89 and G91 show the same type of ligand-induced conformational exchange although they are located on the other side of the protein in turn $\beta$F-$\beta$G and did not exhibit millisecond exchange in the apo-form.

We have considered the possibility that encounter complexes may be nonproductive. Since the binding pocket in apo-CRBP is occluded by the portal region, any conformational state of residues in the portal region different to that in apo-CRBP is likely to allow better access to the cavity. Unfortunately, the resolution of the shoulders in NMR signals is not sufficient to distinguish between signals corresponding to conformers which allow ligand binding vs others which do not. However, line shapes allow a qualitative conclusion whether a cluster of signals includes conformers which are productive and therefore in direct exchange with the signal of holo-CRBP. In the case of fast exchange between intermediates and the holo-protein, this is straightforward because signals in 2D spectra move on a line between two states. For slow exchange, a similar conclusion is possible from the fact that the intensity of signals must become smaller for subsequent steps of the titration if these signals are associated with productive binding. Otherwise, we would expect that conformers exist at a similar intensity during the titration and possibly even in the complex. Since this is not the case for any of the lines associated with mechanism (3), we can conclude that a substantial amount of the $CRBP^*_i$ conformers must be productive.

Figure 3 classifies residues with respect to their participation in a defined step of the binding process. Residues involved in the first high off-rate interaction of retinol with apo-CRBP are displayed red in panel a. Since most of them point away from the $\beta$-barrel into the solvent, they are ideally suited for initial contact with the ligand. Many of these residues, particularly those in the portal region also displayed millisecond mobility in the apo-protein.[30] Furthermore, some of these residues located in the portal region showed slow conformational averaging (green in Figure 3b) for higher concentrations of retinol. This combination of events points to the links between the internal dynamics of the apo-protein and its transformation to slow conformational processes which are required to allow for ligand entry into the $\beta$-barrel, very likely because in some conformers the cavity becomes accessible. This interpretation is supported by the observation that most residues which show slow exchange phenomena are also involved in an initial low affinity interaction. The additional slow conformational exchange on the bottom of the $\beta$−barrel may reflect motions mediated by the retinol molecule in the cavity.

The data presented in this study suggest an unexpected view of ligand uptake by its carrier. An initial encounter of retinol with the apo-form of CRBP leads to structural changes that then allow specific, high-affinity binding. This initial interaction is characterized by a high off-rate and must be nonspecific in the

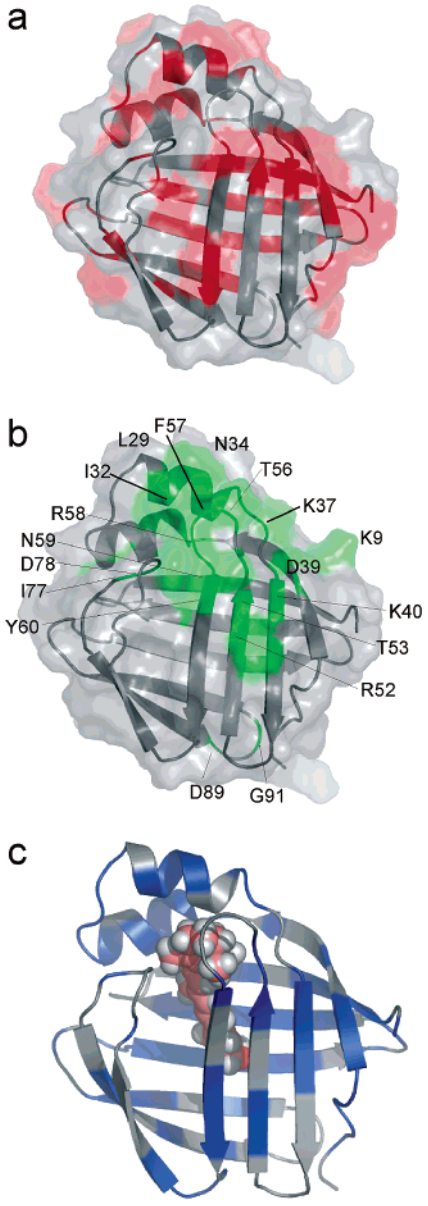


***Figure 3.*** Representations of CRBP depicting residues sensing individual binding steps. (a) Residues labeled red sense a first nonspecific interaction with the ligand as described by eq 2 (residues of this category may also show features of eq 3). (b) Residues labeled green show complex line shapes with shoulders after the addition of small amounts of ligand as described by eq 3 (the same residues may show a first nonspecific interaction as described in eq 2). Most of them line the putative portal, whereas D89 and D91 are located in turn $\beta$F-$\beta$G at the "bottom" of the $\beta$-barrel. (c) Residues in blue show slow-exchange line shapes, which represent the actual high-affinity binding step. Residues in this category may be described by eqs 1−3.

sense that the ligand does not access the binding cavity. It will cause the ligand to be loosely bound to the surface of the protein which may slow the movements of loops in the portal region. Another indication of this kind of nonspecific interaction can be seen in residues that show similar chemical shift changes with a high off-rate when excess retinol was added to purified holo-CRBP: Excess retinol interacts nonspecifically with residues on the outside of the $\beta$-barrel (see the Supporting Information).

The slow time-scale of conformational rearrangements in the portal region of CRBP suggests that unsuccessful binding of the ligand in an unsuitable orientation followed by dissociation may play some role. Although ligand rearrangement is a likely process associated with the proposed mechanism, the location of residues showing slow exchange in the portal region suggests that it is the rearrangements in the protein that control ligand uptake. The binding step that is responsible for the high affinity of the interaction is sensed by many residues all over the protein including those which are part of the $\beta$-barrel and residues in the portal region (Figure 3c).

## 4. Concluding Remarks

This investigation of binding dynamics using line shape analysis of NMR signals presents a highly complex mechanism of retinol uptake by CRBP. In an initial step, the ligand molecules interact nonspecifically with the surface of the protein. Although the interaction is nonspecific it modulates the millisecond dynamics in the portal region thus enabling the ligand to access the cavity. The described mechanism for modulating the accessibility of the binding cavity may play an important role for retinol transport and protection. The advantage of this mechanism is that the retinol binding pocket is not exposed until there is a ligand available to bind to it. This work provides a new perspective on how mobility of a protein has evolved to fulfill its function.

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**Supporting Information Available:** Influence of methanol on apo-CRBP, influence of retinol on holo-CRBP, and a figure that visualizes the mechanism types observed in line shapes across the protein. This material is available free of charge via the Internet at http://pubs.acs.org.

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